

Cloning and analysis of an *Aspergillus nidulans* Sec7
domain coding gene

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ABSTRACT

This study aimed to identify the genetic basis of the *Aspergillus nidulans* *hypB5* mutant phenotype. *A. nidulans* is a filamentous fungus that is widely used as a cell biological and molecular genetic model system. Its hyphae grow by localized polar secretion, producing tubular cells. *A. nidulans* hypercellular strains define five unlinked genes, *hypA1-hypE2*, which cause hyphal morphogenesis defects at 42°C. *hypA* is orthologous to *Saccharomyces* TRS120, which mediates Golgi transit and is widely conserved. The *hypB5* restrictive phenotype resembles *hypA1*: wide hyphae, short basal cells and small nuclei. Like *hypA1*, shifting *hypB5* mutants from 28°C to 42°C causes cessation of tip growth but isotropic expansion of basal cells. A *hypA1, hypB5* double mutant was impaired for growth at 28°C, suggesting these genes have related roles, but neither was epistatic at 37°C so they function in different pathways. The *A. nidulans* pRG3-AMA1 genomic library was used to clone *hypB5* complementing DNA by phenotype rescue, and subcloned to a 5 kb *KpnI* fragment, pYY2. pYY2 was disrupted and sequenced by Tn1000 insertional mutagenesis. The pYY2 sequence is 4975 bp and encodes a putative Sec7 domain which has 81% identity to the *Saccharomyces* SEC7 domain. The Sec7 domain is highly conserved from yeasts to mammals. *Saccharomyces* SEC7 encodes a guanine nucleotide exchange factor involved in COPI vesicle formation and Golgi biogenesis. Insertions in the pYY2 non-Sec7 domain coding region complemented *hypB5* efficiently, whereas those in the Sec7 domain did not, indicating that the Sec7 domain is sufficient for function. A point mutation was found in the *hypB5* strain Sec7 domain, which could explain temperature sensitivity. However, the pYY2 sequence is found on chromosome I whereas *hypB* maps to chromosome VII. Although the origin and functional role of the point mutation in the *hypB5* strain Sec7 protein remains unresolved, it appears that pYY2 contains an extragenic suppressor. Thus *hypB* likely encodes an element in the COPI vesicle assembly pathway.

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LIST OF ABBREVIATION

Chemicals

4-NQO	4-nitro quinoline oxide
amp	ampicillin, D[-]- α -aminobenzylpenicillin
chloro	chloramphenicol, D[-]-threo-2-dichloroaclamido-1-[p-nitrophenyl]-1,3-propanediol
CIP	calf intestinal alkaline phosphatase
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl β -D-thiogalactopyranoside
PABA	p-aminobenzoic acid
PEG-8000	polyethylene glycerol 8000
rif	rifampicin
SDS	sodium dodecyl sulfate (sodium lauryl sulfate)
TAE	Tris-acetate-EDTA
TE	Tris-HCl-EDTA
tetra	tetracycline
Tris	tris[hydroxymethyl]aminomethane
Xgal	5-bromo-4-chloro-3-indolyl- β -galactoside

Media

CM	complete medium (<i>Aspergillus nidulans</i>)
LB	Luria Bertani (<i>Escherichia coli</i>)
MM	minimal medium (<i>A. nidulans</i>)

Vocabulary

AMA1	autonomous maintenance in <i>Aspergillus</i>
ARF	ADP-ribosylation
BFA	brefeldin A
bp	base pair
COP	coatamer protein
DNA	deoxyribonucleic acid
GEF	guanine-nucleotide-exchange factor
kb	kilo base pair
MCS	multiple cloning site
NETO	new end take off
NSF	N-ethylmaleimide sensitive fusion protein
ORF	open reading frame
PCR	polymerase chain reaction
RNase	ribonuclease
SNAP	synaptosome associate protein
SNARE	soluble N-ethyl maleimide sensitive factor attachment protein receptor
TAGKO	transposon-arrayed gene knock-out
UV	ultraviolet
VSC	vesicle supply center

1. INTRODUCTION

1.1 Cell polarity

1.1.1 Overview of cell polarity

Cell polarity is asymmetry in cell shape, protein distribution and/or function. Polarity establishment is required for movement and/or differentiation of cells, so it is ubiquitous except in certain quiescent cells including some unfertilized eggs and ungerminated spores. Polarity is fundamental to morphogenesis and to development of unicellular and multicellular organisms. For example cell polarity is required for 1) positioning of cleavage planes and production of animal and vegetal poles in developing animal embryos (Strome, 1993), 2) epithelial cells that have clearly defined apical and basal compartments, 3) neurons that have spatially separated distinct axonal and dendritic regions (Colman, 1999), 4) polarized plant cells including pollen tubes and root hairs (Kost *et al.* 1999), 5) fungal cells, both unicellular yeasts and filamentous hyphae. Tip growth, polarized cell extension, is the hallmark of fungal hyphae (Heath, 1990), whose structure and functional asymmetry have been studied for over a century. In unicellular fungi, such as the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, polarized growth is controlled by an extensively studied genetic network (e.g., Madden *et al.* 1992, Mata and Nurse, 1998). Analysis of diverse species reveals that cells use conserved pathways to generate cell polarity (reviewed by Anderson and Ingham, 2003). Thus analyses of morphological mutant phenotypes in *Aspergillus nidulans* will contribute to our understanding of conserved mechanisms, as well as the possible variations.

1.1.2 General features of cell polarity of fungi

Cell polarity can be generated from a non-polarized cell, as occurs during germination of an *Aspergillus nidulans* conidium. For this to occur, a region of specialization must be created in the cell cortex, which then is used to direct additional processes that maintain and reinforce the asymmetry. Early aspects of polarization

processes have been studied extensively in the zygotes of brown algae (Belanger and Quatrano, 2000) and in branch initiation in oomycetes (Allaway *et al.* 1997). Thereafter, receptors and signaling proteins mark and interpret the cue. Finally, the asymmetry marked by the cue is transmitted into the cytoplasm and along the plasma membrane. In addition to basic metabolic and synthetic processes required by all cells, polarized growth requires 1) vesicles to transport wall components to the extending tip, 2) a functional actin cytoskeleton (filamentous actin and actin-binding proteins determine support and force-generation) to transport the vesicles and to support the forming tip, 3) cytoplasmic regulatory molecules (ions, second messengers, and the proteins with which they interact).

The unicellular yeast *S. cerevisiae* and *S. pombe* share a number of features of their polarized growth pattern: 1) during their respective cell cycles, both of them have a polarized growth during which cell wall building is restricted to a specific site on the cell surface. In *S. cerevisiae*, the site is cell type specific (haploid α and a mating type cells exhibit an axial pattern while diploid a/α cells bud in a bipolar manner) and is controlled by an internal budding program (Chant, *et al.* 1995). In *S. pombe*, the cells grow from only one tip (the older end) following mitosis, and then when a critical cell length has been achieved (coincident with the onset of S phase), activated growth starts from the second end in a process referred to as new-end take off (NETO) (Mitchison and Nurse, 1985). 2) Polarized growth in both yeasts is seen not only during their vegetative growth but also in response to a mating signal (Chenevert, *et al.*, 1994).

Filamentous fungi like *A. nidulans* establish polarity after breaking spore dormancy and isotropic growth. The germ tube emerges at a site on the cell surface during the early stage of germination. Maintenance of polarized hyphal growth requires continued delivery of secretory vesicles to the growing tip. The overall model of polarized growth in hyphal cells differs from that displayed by the yeast cells. For example, cytokinesis is not accompanied by the onset of a new round of polarization. However filamentous fungal cell polarity is controlled by a well characterized genetic network conserved from unicellular yeasts to multicellular hyphal cells (Harris *et al.* 1997).

The intact actin cytoskeleton is essential for cell polarity in yeasts (reviewed by Pruyne and Bretscher, 2000) as well as in hyphal cells (Harris *et al.* 1994). In contrast,

microtubules are important for cell polarity in fission yeast (Kanbe *et al.* 1994), but are not strictly required for polarity in budding yeast (Yang *et al.* 1997) and filamentous fungi (Xiang *et al.* 1995).

1.1.3 Role of vesicle trafficking in cell polarity

In hyphal tip growth, polarized extension of the cell surface requires the fusion of vesicles with the apical plasma membrane. These vesicle membranes contain integral proteins that must be directed to the proper membrane domain (Brennwald and Adamo, 2000) including those for ion mediated processes, and others for synthesizing wall fibrils. The selection of molecules to be incorporated into exocytic tip growth vesicles is crucial for maintaining polarity. Molecular sorting depends on the basic mechanisms applied to variable cell types: 1) diffusion of membrane components is highly restricted, 2) some proteins may be immobilized by domain-specific interaction with the cytoskeleton, 3) intracellular vesicle trafficking may play a critical role in controlling cell polarity (Burdick *et al.* 1996)

Targeting specific membrane proteins for exocytosis requires 1) generation of a local concentration of those proteins in a donor membrane, 2) vesicle formation from that specialized region, 3) directed vesicle trafficking. This section will focus on the formation of membranous vesicles. The endomembrane pathway consists of a series of distinct membrane-bound compartments interconnected by vesicle trafficking. Nascent proteins intended for delivery to the cell surface are inserted into the membrane or contained in membrane compartments during synthesis on the endoplasmic reticulum (ER) from where they may undergo further modifications before being transported via the Golgi equivalent to their final destination.

An early stage in exocytosis is the budding of the transport vesicles from the donor membrane (such as the ER), then transport via the cytoskeleton and docking on the appropriate receptor membrane (often the Golgi complex or in fungi the Golgi equivalent) followed by fusion with the target organelle. In order to generate the force to form a globular vesicle from a donor membrane, the cell creates a localized membrane associated protein skeleton which in fungi is composed of coatamer proteins, called

COPI and COPII. In animal cells, analogous processes require clathrin and its associated proteins (reviewed by Gorelick and Shugrue. 2001). Vesicle trafficking in the early stage of the secretory pathway requires COPI (Serafini *et al.* 1991) or COPII (Barlowe *et al.* 1994) coat complexes (Fig. 1.1 and 1.2).

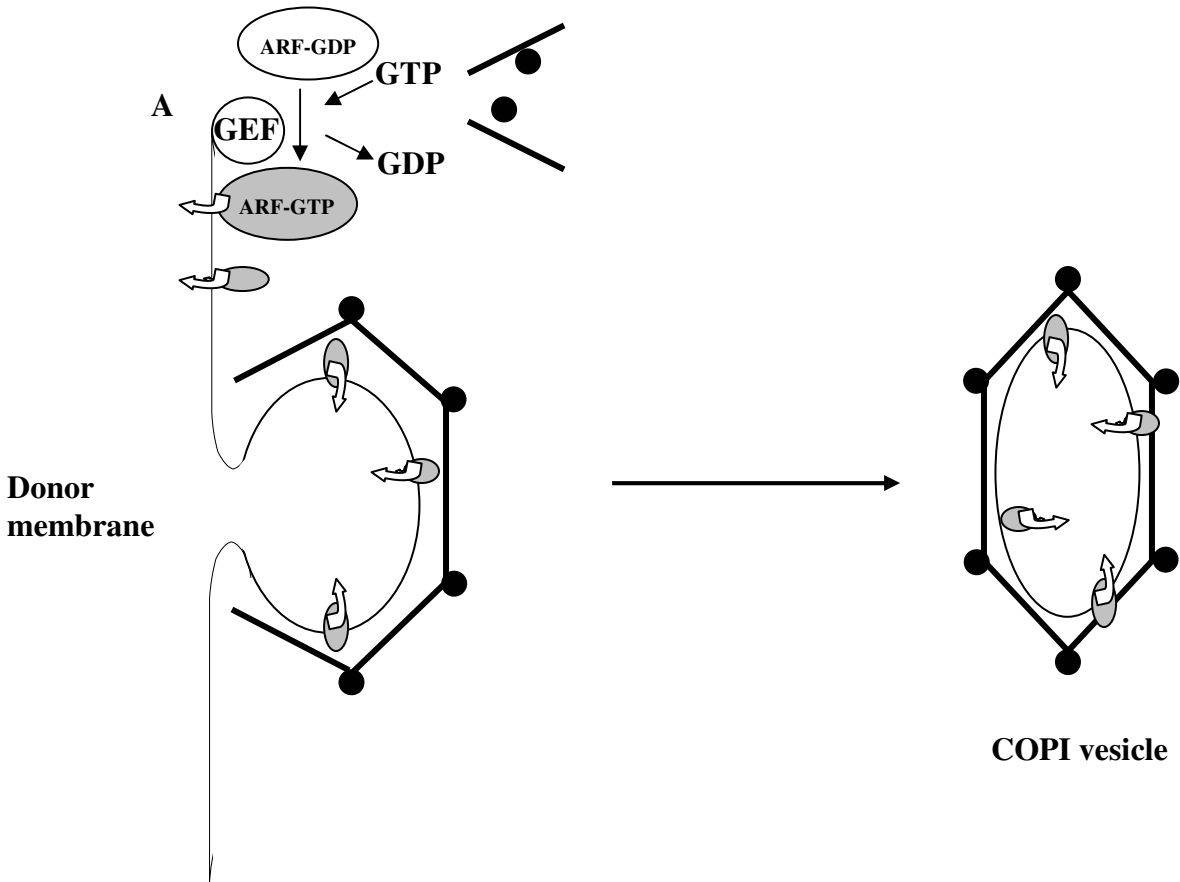


Fig. 1.1 Formation of a COPI coated vesicle. Inactive ADP-ribosylation factor (ARF) is cytosolic and is complexed with GDP. An ARF-specific GTP exchange factor (GEF, which contains a Sec7 domain), catalyzes the exchange of GTP for GDP. Binding GTP creates a conformational change in ARF that exposes an N-terminal myristate (bent arrow), which in turn promotes binding and anchors ARF-GTP to the donor membrane. Once membrane-associated, ARF-GTP recruits cytosolic COPI subunits (seven subunits, shown collectively as circles and rods). The ARF-GTP-COPI complex reshapes the donor membrane into a globular vesicle. Disassembly of the COPI coat (not shown) for vesicle fusion begins with GTP hydrolysis, an endogenous activity of ARF.

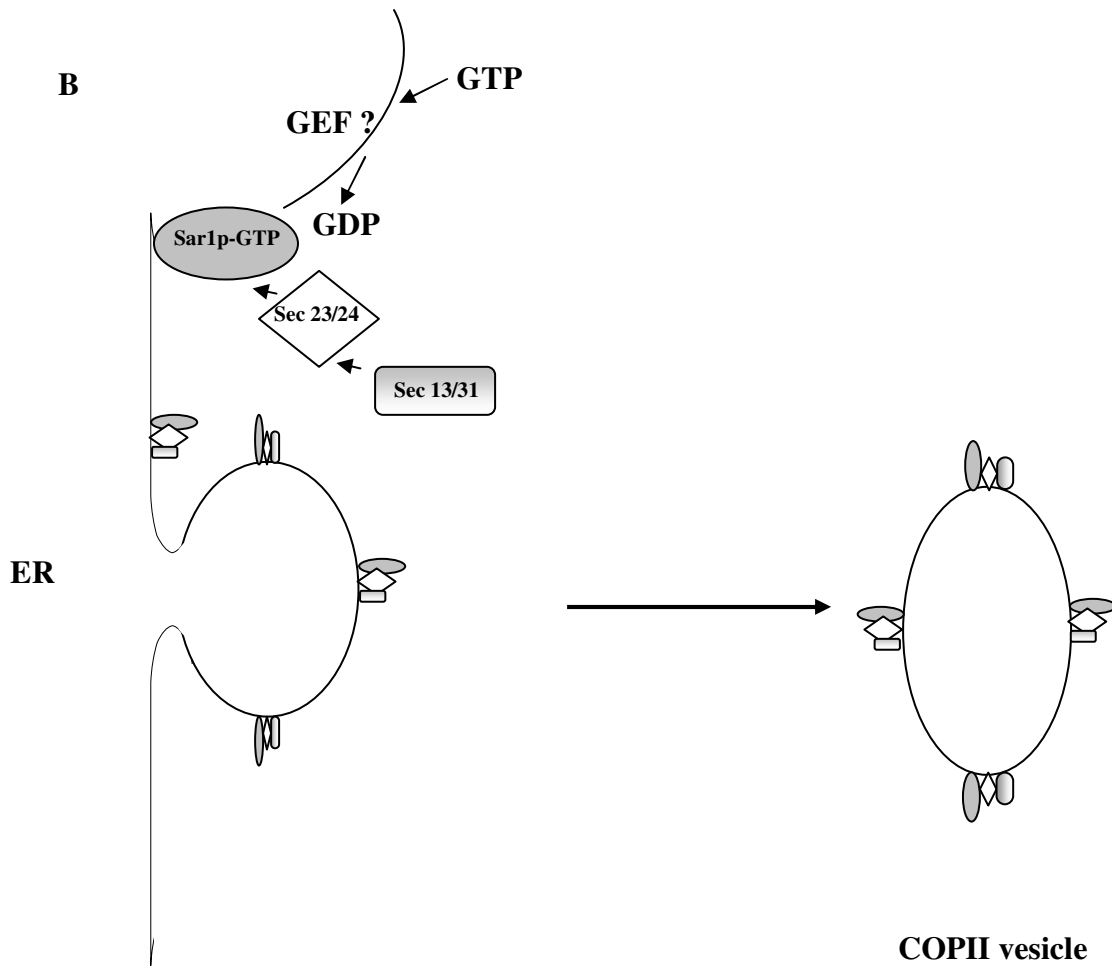


Fig. 1.2 Formation of a COPII coated vesicle. Sar1p-GDP is presumed to be soluble in the cytoplasm and to have a specific GEF that activates its binding to the donor membrane to initiate COPII coat assembly. The members of the COPII coatomer are added sequentially (Shown as $\diamond \square$). Sec 23/24 and Sec 13/31 complexes interact with the ER membrane and induce vesicle bud formation.

The COPI complex contains eight subunits: ADP-ribosylation factor (ARF) and seven coatmer proteins (COPs: α , β , β' , γ , δ , ϵ and ζ ; Gaynor *et al.* 1998). Wild type hyphal morphogenesis requires normal endomembrane vesicle dynamics. For example, the *A. nidulans* sod^{VI} C morphogenetic mutant characterized by Whittaker *et al.* (1999) encodes an α -COPI. In the COPI pathway, coated vesicle formation depends on ARF binding to the donor membrane, which in turn induces vesicular coat assembly by recruiting cytosolic coatmer proteins to reshape the donor membrane into a spherical form. The active GTP bound ARF is catalyzed by Golgi-localized accessory proteins known as guanine-nucleotide-exchange factors (GEFs) to exchange GTP for GDP on inactive ARF. This step is sensitive to the fungal metabolite brefeldin A (BFA) (Sata *et al.* 1999). Without GEFs, GDP-GTP exchange takes place slowly. When ARF binds GTP, a conformational change exposes the N-terminally bound myristic acid of ARF, after which ARF-GTP binds to the membrane to initiate coat assembly. Chardin and coworkers (1996) showed that GEF's activities reside within the Sec7 domain, which is exclusively found in the Sec7 protein family, and that the Sec7 domain alone is sufficient for GTP nucleotide exchange activity. Presumably, substrate specificity (ARF vs others) resides in the non-Sec7 protein domain(s). ARF is also required for vesicle uncoating by hydrolyzing GTP (Tanigawa *et al.* 1993). Uncoating is required for vesicle fusion with the target membrane.

The production of COPII coated vesicles is achieved by neutral phospholipid dependent interaction of the GTP bound form of Sar1p, which recruits two large heterodimeric complexes, Sec23/24 and Sec13/31, to the ER membrane (reviewed by Haucke 2003). COPII coated vesicle biogenesis is not affected by BFA. Independent ontogeny of the two kinds of coated vesicles and their differential sensitivity to BFA suggests they may mediate distinct transport routes from ER to Golgi.

Genetic, biochemical and morphological studies in yeast and mammalian cells have defined a role for COPII-coated vesicles in anterograde trafficking of proteins from the ER to Golgi (Rothman and Wieland, 1996). In contrast, the role of COPI-coated vesicles trafficking in the early secretory pathway is not fully understood. Bednarek *et al.* (1995) implicated COPI-coated vesicle transport from ER to Golgi. Letourneur *et al.* (1994)

suggest COPI may also mediate post-ER retrograde trafficking due to its coatamer binding with the di-lysine retrieval motif (KKXX), a signal which is required for retrograde movement from post-ER back to the ER. The anterograde COPI-coated vesicle traffic may have different protein and lipid content from those of COPII-coated vesicles. The retrograde traffic may involve intracellular recycling of cellular materials.

The interaction between integral membrane proteins on the vesicles, called vesicle-soluble N-ethyl maleimide sensitive factor attachment protein receptors (v-SNAREs) with the corresponding proteins on the target membranes, called t-SNAREs ensures correct loading of vesicular contents to their destined target membrane. Vesicle fusion also requires soluble proteins that recognize the SNARE complex, termed synaptosome associated proteins (SNAPs) and N-ethylmaleimide sensitive fusion proteins (NSF) (reviewed by Rothman, 1994; Gupta and Heath, 2002).

1.2 The role of *Aspergillus nidulans* in biological and genetic study

The aspergilli are filamentous fungi that play an important experimental role in cell biology and molecular genetics, and link unicellular and multicellular organisms (Smith and Pateman, 1977). *Aspergillus nidulans* is the asexual state of an ascomycete fungus, which produces a simple multicellular vegetative mycelium. It is an excellent system for studying cell biology and cellular development since: 1) it is eukaryotic, so its genetic regulation is closely related to that of animals and plants, 2) it has a short life cycle, and can complete its asexual life cycle within two days, 3) it has simple nutritional requirements, 4) its differentiation stages have distinctive growth modes (hyphae, conidia and sexual organs), 5) its vegetative state is haploid, so the recessive mutations are readily identified, 6) the sexual stage, *Emericella nidulans* is readily induced. Taken together these features led to *A. nidulans* being adopted as a model system for molecular genetics and cell biology.

A. nidulans has eight chromosomes. The genome is about 31 million base pairs, containing 11,000-12,000 genes; the draft sequence of *A. nidulans* is available at www-genome.wi.mit.edu/media/2003/pr_03_aspergillus.html. The genome sequence contains 2-3% repetitive DNA (Timberlake, 1978), which are short, low copy,

interspersed repeats (Brody and Carbon, 1989). By classical genetic analysis, about 900 loci have been assigned to its eight linkage groups (<http://www.gla.ac.uk>), at least 432 have been mapped to locus, and 254 are cloned and sequenced.

A. nidulans genes can be cloned by complementation of mutations by using cosmid, plasmid, or one of several genomic libraries (Johnstone *et al.* 1985). By rescuing the transforming plasmids from *A. nidulans* into *Escherichia coli*, the complementing activities can be distributed rapidly to small restriction fragments (Timberlake, 1985).

1.3 Hyphal morphogenesis in filamentous fungi

1.3.1 General features of hyphal tip growth

Filamentous fungi grow by apical extension of multinucleate cells called hyphae, which are later subdivided by the insertion of cross walls called septa. Hyphal tip growth depends on the deposition of new wall materials at the tip (reviewed by Kaminskyj and Heath, 1996). Most materials are transported in Golgi derived membrane-bound vesicles from the subapical areas, and their insertion into the tip membrane is precisely controlled to generate cylindrical hyphae. The variation of the position and/or the rate of vesicle insertion will affect hyphal shape (Bartricki-Garcia, 2002).

1.3.2 Mechanisms regulating hyphal morphogenesis

Factors that affect hyphal morphogenesis including cytoplasmic ion gradients, notably calcium (Jackson and Heath 1993), hydrogen (Bachewich and Heath 1997), and manganese (Sone and Griffiths 1999); second messengers such as cAMP (Bruno *et al.* 1996); and regulating gene products such as *Ras* (Som and Kolaparthi 1994) and Rho-type GTPases (Wendland and Philippsen 2000). Cytoskeletal filaments and actin-based and microtubule-based motor proteins are required for the hyphal polarity establishment and maintenance (McGoldrick *et al.* 1995; Wu *et al.* 1998). Intermediate filaments called septins are also required for polarization (Momany *et al.* 2001, Harris *et al.* 1997). Hyphal morphological features are

consistent for a given species, growth stage and growth condition but different from one species to another, even within the life cycle of a single species, so the tip growth process is capable of subtle modification.

1.3.2.1 Spitzenkörper

The Spitzenkörper (tip body) is a dynamic assemblage of vesicles found in tips of growing hyphae in many higher fungi. Girbardt first linked the Spitzenkörper with the growth of hyphae in 1969. Bartricki-Garcia *et al.* (1995) have used computer models to simulate the role of a theoretical vesicle supply center (VSC), whose position is coincident with Spitzenkörper. In this model, the wall-building vesicles are randomly directed to the growing tip from the Spitzenkörper creating tip high gradient of vesicle insertion. Along with the extension of growing hyphae, concomitant apical migration of the VSC is thought to shape the growing hypha. The dynamics of Spitzenkörper size and activity can determine the direction of tip growth as well as hyphal shape. The forced movement of Spitzenkörper by laser microbeam can induce an aberrant hyphal shape at the site where the cell wall is adjacent to the new position of the VSC, or the hyphal growth is redirected even subsequently generates a new branch (Bracker *et al.* 1997).

1.3.2.2 The Cytoskeleton

The cytoskeleton is a network of protein filaments and their accessory proteins. It is a dynamic, three-dimensional structure that fills the cytoplasm. The cytoskeleton maintains the cell's shape in areas where cell wall is newly deposited and not yet fully crosslinked. The primary types of fibers comprising the cytoskeleton are actin microfilaments, microtubules, and intermediate filaments.

The actin cytoskeleton forms a three-dimensional proteinaceous network, so that the fine structure of cytoplasm is gel like and apart from small dissolved solutes, all cellular components are actively transported. Actin-based transportation which is required for polarized morphogenesis uses the motor protein myosin, powered by ATP hydrolysis (Mulvihill and Hyams, 2003). The controlled vesicle insertions at

the tip, in conjunction with turgor bearing network regulating hyphal extensibility, produces cylindrical hyphae (Gupta and Heath, 1997).

The basic structural unit of microtubules is an alpha- and beta-tubulin heterodimer. Long range transportation of vesicles to the hyphal tip is presumed to be microtubule-dependent (Steinberg and Schliwa, 1993), whereas actin filaments are used to transport vesicles from the Spitzenkörper to specific sites for cell wall deposition. Microtubule disruption by antimicrotubule drugs or inducing conditional microtubule mutants leads to decreased microtubule dynamics (Han *et al.* 2001, Minke *et al.* 2000), but not the aberrant hyphae (Xiang *et al.* 1995). As a result, microtubules are not strictly required for tip extension (Heath, 1990).

The intermediate filaments called septins function during cell polarity establishment (Cid-Victor *et al.* 2001) as well as polarity maintenance by specifying a boundary between cortical domains of budding yeast (Barral *et al.* 2000). Momany and Hamer (1997a) characterized that *A. nidulans* septin filaments are essential for growth. Septins are involved in vesicle trafficking in mammalian cells (Kartmann and Roth, 2001) but this has not been observed in filamentous fungi.

1.3.2.3 Morphogenetic genes

Conditional mutations are those expressed only under certain conditions, for example temperature sensitive defects. These provide a convenient way to observe loss of gene function under specific conditions. Many temperature sensitive conditional *A. nidulans* mutants have been isolated from the mutant collections described by Morris (1976), Harris *et al.* (1994) and Kaminskyj and Hamer (1998). At permissive temperature (28°C), these mutant strains resemble wild type, whereas at restrictive temperature (42°C), they show morphological defects. These defects can be grouped into classes including nuclear division, nuclear migration and hyphal morphogenesis. The latter includes: loss of ability to form septa (*sepA-D*; Harris *et al.* 1994), polarity-defective (*podA-D*; Harris *et al.* 1999; *podG, H*; Osheroov *et al.* 2000a), swollen (*swaA-H*; Momany *et al.* 1999), hypercellular (*hypA-E*; Kaminskyj and Hamer, 1998) and hyperbranch (*hbrA*; Memmott *et al.* 1999).

Hyphal morphological defects are defined by inability to produce wild type hyphae. A severe form is inability to germinate. However this can be due to a fundamental metabolic defect (Osherov *et al.* 2000b). podHp shares identity with yeast TFIIF interacting component of CTD phosphatase (Osherov *et al.* 2000a). *swoH* encodes protein with nucleotide diphosphate kinases (Lin *et al.* 2003). In addition, podGp is a homologue of yeast α subunit of mitochondrial phenylalanyl-tRNA synthetases (Osherov *et al.* 2000a). More germane defects include those related to cytoskeleton, endomembrane communication or wall formation, including *sepA*, *swoA*, *swoF* and *hypA*. Depending on their identity, morphogenetic genes may be involved at different stages, function on specific substrates or act as the subunit of a functional complex. hypAp is involved in polarized growth most likely by mediating Golgi transit (Shi, Sha and Kaminskyj, submitted).

1.4 Wild type *Aspergillus nidulans* morphologies

A. nidulans spores are triggered to germinate under environmental conditions which provide oxygen, water and a reduced carbon source (d'Enfert, 1997). The spore isotropic growth is followed by the emergence of a germ tube from a localized point on the cell surface when the first round of nuclear division is completed (Harris *et al.* 1994). After that hyphal extension only occurs at the extreme tip. In a growing hypha, mitotically active nuclei are located in the tip cells or branched basal cells, whereas nuclei are quiescent in unbranched basal cells. *A. nidulans* hyphae are ~ 3 μm in width, and average ~ 40 μm in length for the basal cells (Kaminskyj and Hamer, 1998). First septum is deposited after three rounds of nuclear division at the base of the germ tube (Harris *et al.* 1994). Septation depends on mitosis, cell size and nuclear positioning (Momany and Hamer, 1997 b). Each basal cell contains 3-4 evenly spaced nuclei. Unlike basal cells, tip cells are variable in their length and number of nuclei.

1.5 *hyp* mutations of *Aspergillus nidulans*

In screening an *A. nidulans* temperature sensitive conditional mutant collection, five strains with defective morphogenesis were identified and named *hyp* for hypercellular (Kaminskyj and Hamer, 1998). Strains carrying a *hyp* mutation are conditionally defective in growth pattern, but do not have a lethal temperature sensitive arrest. Segregation after classical genetic analysis showed that each strain was due to a single gene defect and all five gene loci were independent. As a group, the *hypA-hypE* strains have abnormal restrictive phenotypes including wide, slow growing hyphae with short subapical cells with abundant nuclei. Phenotypic analysis showed that *hyp* mutants display defects in polarity establishment, tip growth and septation.

1.5.1 *hypA*

When *hypA1* mutant cells are grown at restrictive temperature (42°C), the germlings germinate and grow slowly, and produce significantly wider hyphae than at permissive temperature (28°C). However they have normal nuclear division cycles, and are able to complete their asexual life cycle, conidiating at the restrictive temperature after a slight delay, producing viable spores. *hypA* was mapped to chromosome I and encodes a predicted protein of 1474 amino acids with molecular weight of 161.9 kDa (Shi, Sha and Kaminskyj, submitted). The *hypA1* allele is caused by a point mutation creating an arginine at 329 instead of glycine (G329R). The orthologue of *hypA* in *S. cerevisiae*, TRS120, is a putative regulatory subunit in the TRAPP II complex mediating Golgi trafficking by COPII vesicle pathway. *S. cerevisiae* TRS120 is essential (Sacher *et al.* 2000), but surprisingly *A. nidulans hypA* is not (Shi, Sha and Kaminskyj, submitted), which is consistent with its putative regulatory role. As expected given its homology to TRS120, the endomembrane arrays of *hypA1* strains are substantially compromised at restrictive temperature. *hypA1* strains have a novel phenotype when 28°C growing cells are shifted to 42°C. Their basal cells, but not their tip cells, restructure and swell to the restrictive diameter. Strains expressing the *hypB5* mutation have similar restrictive

and upshift phenotypes to *hypA1*, but these are distinct genes and the double mutant has a growth defect even at 28°C, suggesting they are in a related pathway. As a result, *hypB* was selected for analysis and cloning as discussed below.

1.5.2 Restrictive phenotype of *hypB5*

When grown at 42°C, *hypB5* germlings have significantly wider hyphae and shorter subapical cells than wild type, as in the case of *hypA1*. The increase of hyphal width and decrease of basal cell length may maintain a constant basal cell volume. However, the mechanism that determines septum spacing in *A. nidulans* is still unclear (Kaminskyj, 2000). At the restrictive temperature, the shorter subapical cells of *hypB5* strains have significantly more nuclei than the typical 3-4 nuclei per cell in wild type hyphae. In addition, 42°C growing *hypB5* germlings have abnormalities, including near apical, lateral branches and some anucleate basal cells (Kaminskyj and Hamer, 1998).

Compared to wild type, *hypB5* strains exhibit delayed germination even at the permissive temperature, which was exacerbated at restrictive temperature. This germination delay may be due to the defects of *hypB5* strains in cell polarity establishment. Septum formation was delayed compared to wild type when *hypB5* strains were grown at 42°C, and occurred in short cells containing ≥ 16 nuclei (Kaminskyj and Hamer, 1998).

1.6 Objectives

1. To complement the *A. nidulans hypB5* morphogenesis defects using a wild type genomic library. Complementation will be shown by wild type colony growth at 42°C, presumed to be the gene of interest or a multicopy suppressor that interacts with the mutant allele. The smallest complementing DNA fragment will be cloned and used for further characterization.

2. To sequence the *hypB5* complementing DNA and to analyze it to suggest likely function and to guide further research. Sequencing will use a transposon mediated

insertional mutagenesis. Analysis will begin with BLASTX and Gene Ontology (GO) studies (Ashburner *et al* 2000).

3. To determine whether the *hypB5* complementing DNA likely encodes *hypB* or a suppressor. This will employ morphometric comparison of the rescued, wild type, and *hypB5* strains, and bioinformatics. Whether *hypB* or a suppressor is cloned, the complementing DNA will be relevant to hyphal morphogenesis. *hypB* has been mapped genetically, and if the complementing DNA sequence is found in that chromosomal region, this will provide evidence that the gene cloned is the gene of interest. Otherwise, this will suggest that the cloned gene may be a suppressor of the gene of interest.

4. To identify the lesion in the *hypB5* mutant likely to be responsible for the temperature sensitive phenotype. The complementing DNA containing transposon mediated disruptions will be used to identify regions essential for complementing activity. Sequencing this region in the *hypB5* strain and comparing it to the wild type strain is expected to reveal the mutation site.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Biological materials

Table 2.1 lists the *Aspergillus nidulans* strains, the pRG3-AMA1 *A. nidulans* genomic library and plasmids used in this research. The multiple cloning site of pBCKS+ (www.stratagene.com) is shown in Figure 2.1.

2.1.2 Solutions, enzymes and primers

The formulation of each solution used in this research is listed in Table 2.2

Restriction endonucleases and T4 DNA ligase were purchased from Gibco BRL (www.lifetech.com). *Taq* DNA polymerase was purchased from Amersham Pharmacia Biotech (www.apbiotech.com).

PCR primers are listed in Table 2.3. They were synthesized by Life Technologies (www.lifetech.com) or by Cortec (www.cortec.ca).

2.1.3 Chemicals

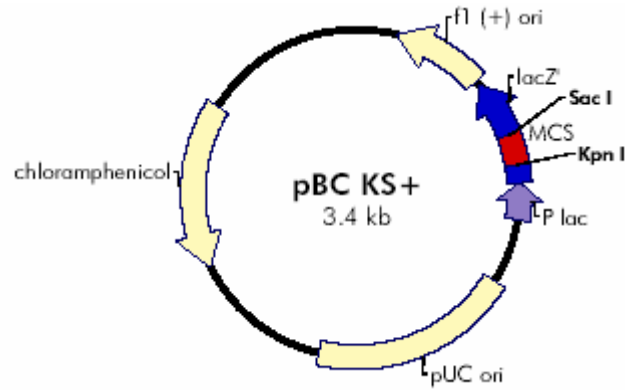
All chemicals used were reagent grade and purchased from VWR (www.vwrsp.com) or Sigma (www.sigmaaldrich.com) unless otherwise noted. All water used was deionized to 18 Meg Ohm or double distilled, and was sterilized as appropriate.

Table 2.1 Biological materials used in this study

<i>Aspergillus nidulans</i> strains	Genotype
A28 ^a	<i>biA1, pabaA6; veA1</i>
ASK80 ^b	<i>pabaA6; hypB5; chaA1, veA1</i>
ASK166 ^b	<i>pyrG89; wA2; hypB5; veA1</i>
AYY1 ^c	<i>hypB5</i> complemented strain, <i>wA2; veA1</i>
ASH83 ^f	<i>pabaA6; podB1; pyroA4; veA1</i>
Ts 1-160 hbrA ^g	<i>biA1, pabaA6; hbrA; veA1</i>
<i>Escherichia coli</i> strains	
XL-1 blue ^d	Tetracycline ^R
DH5α ^d	
pRG3-AMA1 <i>A. nidulans</i> genomic library ^e	Ampicillin ^R and <i>Neurospora crassa</i> <i>pyr4</i>
Plasmids	
pBCKS+ ^d	pBluescript, chloramphenicol ^R
pYY1 ^c	plasmid rescued from pRG3-AMA1 <i>A. nidulans</i> genomic library by complementation of <i>hypB5</i> defective phenotype at restrictive temperature, Amp ^R
pYY2 ^c	5 kb <i>KpnI</i> digested pYY1 fragment inserted into pBCKS+, chlor ^R

(a) Fungal Genetics Stock Center (www.fgsc.net), (b) Kaminskyj and Hamer (1998), (c) generated in this study, (d) Stratagene (www.stratagene.com), (e) a gift of Dr. Osharov, N and May, G., (f) Harris *et al.* (1999), (g) Memmott and Turnor (1999)

A



B

TTGTAAAACGACGGCCAGTGAGCGCGGTAATACGACTCACTATAGGGCGAAT TGGAGCTCCACCGCGGTGGC
 T7 primer binding site

GGCCGCTCTAGAACTAGTGGATCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGA

GGGGGGGCCC GGTACCCAGCTTTTGTTC CCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCATAGC
 KpnI
 T3 primer binding site

TGTTTCC

Fig. 2.1 The schematic structure of (A) PBCKS+ (Stratagene, www.stratagene.com) which contains chloramphenicol resistance marker and *lacZ* embedded in its multiple cloning site (MCS). (B) The PBCKS+ Multiple cloning site (sequence from 653 to 760). *KpnI* cutting site is located within the MCS at the position 755.

Table 2.2 Solution formulations

Solutions	Formulation
5X T4 DNA Ligase buffer	250 mM Tris-HCl (pH 7.6), 50 mM MgCl ₂ , 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000
10X Taq DNA polymerase buffer	100 mM Tris-HCl (pH 8.0), 50 mM NH ₄ Cl, 15 mM MgCl ₂ , 500 mM KCl
TAE buffer	40 mM Tris-acetate, 2.2 mM EDTA di-sodium salt (pH 8.0)
TE DNA resuspension buffer	1 mM EDTA in 10 mM Tris, pH 8.0
PEG 8000	20% polyethylene glycol (8000), 2.5 M NaCl
TNK50 PCR buffer	100 M Tris-HCl (pH 8.0), 50 mM NH ₄ Cl, 15 mM MgCl ₂ , 500 mM KCl
5X Cycle Sequencing buffer	400 mM Tris-HCl (pH 9.0), 10 mM MgCl ₂
10X PCR buffer #8	10 mM Tris-HCl (pH 8.8), 75 mM KCl and 3.5 mM MgCl ₂
1X Fixative	3.7% formaldehyde, 50 mM phosphate (pH 7.0), 0.2% Triton-X100
Mounting buffer	50 mM sodium phosphate (pH 8.0), 50% glycerol, 0.1% n-propyl gallate
RNA extraction buffer	150 mM LiCl, 5 mM EDTA, 50 mM Tris-HCl (pH 9.0), 5% SDS and 0.1 M 2-mercaptoethanol
20 X SSC (pH 7.2)	3 M NaCl, 0.3 M sodium citrate
cDNA synthesis buffer	250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl ₂
<i>A. nidulans</i> transformation solutions	
Solution I	0.8 M ammonium sulphate, 100 mM citric acid
Solution II	0.4 M ammonium sulphate, 2% sucrose, 20 mM MgSO ₄
Solution III	0.4 M ammonium sulphate, 1% sucrose, 50 mM citric acid
Solution IV	25% PEG8000, 100 mM CaCl ₂ , 0.6 M KCl, 10 mM

	Tris-HCl (pH 7.5)
Solution V	0.6 M KCl, 50 mM CaCl ₂ , 10 mM MOPS
<i>A. nidulans</i> genomic DNA extraction solutions	
Solution A	10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.5% SDS
Solution B	RNaseA 10 mg/ml in 50 mM sodium acetate (pH 4.5) heated in boiling water for 5 mintues

Table 2.3 Primers used in PCR and Sequencing reactions

Primers	Sequence (5'-3')	T _m (°C) ^e	Supplier
GDIR ^a	TTTCGTTCCATTGGCCCTCAAACCCC	78	Gibco BRL ^c
GD1 ^a	CAACGAATTATCTCCTT	56	Gibco BRL
GD2 ^a	TCAATAAGTTATAACCAT	51	Gibco BRL
T3	AATTAACCCCTCACTAAAGGG	64	Gibco BRL
T7	GTAATACGACTCACTATAGGGC	69	Gibco BRL
pYY2 241-264 ^b	GAATGACAGCAAGAATAACCTGC	56.2	Cortec ^d
pYY2 488-508	GCAACCTTCGCTACCAATCGG	53.7	Cortec
pYY2 1865-1888	AGCCATACTCGAGAAGCGAAATGC	57.3	Cortec
pYY2 2334-2351	ACAACGTAGCGTTGACCC	49	Cortec
pYY2 2646-2665	ACTTCGCAATGACCGCTTAG	52.3	Cortec
pYY2 3160-3179	CAAGTCTTCGCAACGGTTGG	52.3	Cortec
pYY2 3513-3497	ACTTCGCCAAGGCAGTG	47	Cortec
pYY2 3179-3160	CCAACCGTTGCGAAGACTTG	52.3	Cortec
pYY2 2657-2635	TCATTGCGAAGTATGAAAGCTGC	56.2	Cortec
pYY2 1889-1867	GCATTTTCGCTTCTCGAGTATGG	55.0	Cortec

a. Strathmann *et al.* 1991

b. pYY2 primers with forward numbering are the complement to the forward strand, backward numbering to the reverse strand.

c. Gibco BRL (<http://www.invitrogen.com/>)

d. Cortec (www.cortec.ca)

e. All T_ms were supplied by the synthesis companies.

2.1.4 Media

Media formulations used in this study are listed in Table 2.4 Media for *A. nidulans* growth were prepared according to Kaminskyj (2001). Complete media (CM) supports strains without nutritional auxotrophies, which were supplemented as required. Nitrate salts, trace elements and vitamin solution were prepared according to Kaminskyj (2001).

LB (Luria Bertani) broth was used to grow *E. coli*. Antibiotics were supplemented to select for resistance markers. IPTG and X-gal were added to induce and test *lacZ* expression.

Table 2.4 Formulations of culture media

Species	Media	Formulation
<i>A. nidulans</i>	CM	1% D-glucose, 0.2% peptone, 0.1% yeast
	(complete medium) ^a	extract, 0.1% casamino acids, 0.1% nitrate salts, 0.1% trace elements, 0.01% vitamin solution pH 6.5
	MM	1% glucose, 0.1% nitrate salts, 0.1% trace elements,
	(Minimal medium)	0.01% thiamine, 25 µg/L biotin, pH 6.5
	Bottom agar	CM, 1 M sucrose, 1.5% agar
	Top agar	CM, 1 M sucrose, 0.75% agar
<i>Escherichia coli</i>	LB	1% tryptone, 0.5% yeast extract, 0.5% NaCl
	LB+amp	LB+ 100 µg/ml ampicillin
	LB+chlor	LB + 100 µg/ml chloramphenicol
	LB+tet	LB + 100 µg/ml tetracycline
	LB+rif	LB + 100 µg/ml rifampicin
	LB+IPTG+Xgal	LB + 32 µg/ml of IPTG ^b and X-gal ^b
	SOC	2% tryptone, 0.5% yeast extract, 8.6 mM NaCl, 20 mM glucose

a. For all supplements see Table 2.5

b. Jersey lab supply (www.labscientific.com)

Table 2.5 Formulation of *Aspergillus nidulans* media supplements

Supplements	Formulation
20X Nitrate salt	1.4 M NaNO ₃ , 140 mM KCl, 42mM MgSO ₄ .7H ₂ O, 22 mM KH ₂ PO ₄
1000X Trace element	76.5 mM ZnSO ₄ .7H ₂ O, 178 mM H ₃ BO ₃ , 25.3 mM MnCl ₂ .4H ₂ O, 18 mM FeSO ₄ .7H ₂ O, 7.1 mM CoCl ₂ .6H ₂ O, 6.4 mM CuSO ₄ .5H ₂ O
vitamin solution	0.01% biotin, 0.01% pyridoxine, 0.01% thiamine, 0.01% riboflavin, 0.01% PABA (p-aminobenzoic acid), 0.01% nicotinic acid
200X arginine	4.2 % arginine
20000X biotin	0.05 % biotin
1000X PABA	1 % PABA(para-amino benzoic acid)
20000X pyridoxine	0.1 % pyridoxin
50X pyrimidine	5.6 % uracil, 6.1 % uridine

2.2 Methods

2.2.1 *hypB5* complementing DNA cloning

2.2.1.1 Plasmid DNA preparation

Plasmid DNA was extracted following the protocol of Sambrook and Russel (2001), from *E. coli* overnight culture in 10 mL LB with the appropriate antibiotics. DNA was extracted using the alkaline lysis method followed by two rounds of phenol/chloroform extraction, and finally isopropanol precipitation. The pellet was resuspended in TE buffer and 1/10 volume of 3 M sodium acetate and 2.5 volume of 100% ethanol. After centrifuge, the DNA pellet was washed with 70% ethanol and air dried. The purified DNA was resuspended in 30 μ L TE buffer or distilled water and kept at -20°C.

2.2.1.2 *Aspergillus nidulans* storage, growth conditions

For routine use *A. nidulans* spores were stored in distilled water at 4°C. Spores can be stored in 15-20% glycerol at -80°C for a long period of time (Kaminskyj, 2001). Spores are able to germinate in appropriate media with nutritional supplements, where needed to compensate for an auxotrophy (see Table 2.4 and 2.5 for *A. nidulans* media formulation).

2.2.1.3 *Aspergillus nidulans* transformation

About 10⁹ freshly harvested ASK166 spores were germinated in 50 mL CM liquid with appropriate supplements and 100 μ g/mL ampicillin in a sterile 250 mL Erlenmeyer flask coated with Gel Slick[®] (FMC BioProducts, www.bioproducts.com) followed by incubation at 30°C with shaking at 300 rpm for 6 hours. Germlings were partially protoplasted with 0.6% β -D-glucanase (Interspex, www.wene.net/~sharma/interspex), 0.25% Driselase (Interspex) and 0.25% β -glucuronidase (Sigma). Protoplasts were stabilized with osmoticium (0.2% bovine albumin, 20 mL Solution I and 20mL Solution II) (See Table 2.2). Partial protoplasting required two hours incubation at 30°C, shaken at 150 rpm. Protoplasts were washed twice with cold Solution III (See Table 2.2) and resuspended in 1 mL cold Solution V (See Table 2.2). For transformation, 100 μ L protoplast suspension was mixed with 1 μ g DNA and 50 μ L Solution IV (See Table 2.2)

and incubated on ice for 20 minutes. One milliliter Solution IV was added and the mixture was incubated at room temperature for 20 minutes. Each transformation used about 10^8 protoplasts in a final volume of 1200 μ L. After transformation, three aliquots of 400 μ L from each protoplast suspension were mixed with 4 mL 47°C top agar and plated over the bottom agar.

For *hypB5* complementation, ASK166 protoplasts were transformed with 1 μ g pRG3-AMA1 based *A. nidulans* genomic library (Oshero *et al.* 2000a, 2000b). Transformants were plated on the selection media (CM without added pyrimidines) and incubated at 28°C for 18 hours before being transferred to 42°C. Wild type colonies were isolated two days later. One wild type transformant was named strain AYY1.

2.2.1.4 *Aspergillus nidulans* genomic DNA preparation

Genomic DNA from *hypB5* transformants was prepared following the advice of G. S. May (personal communication). It is critical to maintain the selection pressure since the plasmid containing the genomic insertion of interest can be lost at a high frequency. AYY1 spores were inoculated in 50 mL CM liquid with 100 μ g/mL ampicillin followed by incubation at 42°C for 48-72 hours. Mycelia were harvested by pouring through sterile cheesecloth, frozen at -80°C, and lyophilized for 24 hours in a Labconco Freezone6. About 50 μ L freeze dried mycelia were ground to a powder, to which was added 700 μ L Solution A (See Table 2.2) preheated to 65°C. Dried mycelia were dispersed by vortexing. The supernatant was incubated at 65°C for 30 minutes, and then extracted once with phenol and once with phenol/chloroform. The DNA was recovered using ten minutes spins at 13000 rpm. Ten microliter Solution B (See Table 2.2) was added to the DNA suspension, incubated at 37°C for 30 minutes followed by extraction with phenol/chloroform and then with chloroform. Seventy microliter 3 M sodium acetate was added together with 700 μ L ethanol, mixed by vortexing and incubated at room temperature for 5 minutes. High molecular weight DNA was pelleted by spinning at 15000 rpm for 5 minutes. The DNA pellet was washed with 70% ethanol, air dried and resuspended in 100 μ L distilled water.

2.2.1.5 Preparation of Electrocompetent *E. coli*

A single 12 hours old colony of XL-1 Blue was inoculated in 10 mL LB in a 50 mL sterile conical tube with 100 µg/mL tetracycline at 37°C and shaken at 250 rpm overnight. The overnight culture was transferred to 1000 mL sterile preoxygenated LB in a 2000 mL flask and grown to an OD₆₀₀=0.6 as measured by spectrophotometer (Beckman DU7400, www.beckmancoulter.com). The culture was divided between prechilled 250 mL centrifuge bottles and incubated on ice for 15 minutes. The cells were pelleted in a 4°C AvantiTM J-25 rotor (Beckman) at 5000 x g for 15 minutes. The cells were washed twice with ice cold water then in 10% ice cold sterile glycerol. Aliquots of 40 µL of washed cells were frozen in liquid nitrogen and then stored at -80°C. The cells were spread on LB plates with distinct antibiotics to test contamination. XL1 Blue cells should grow on LB agar and LB plus tetracycline, but not on LB containing ampicillin or chloramphenicol.

2.2.1.6 Electrocompetent *E. coli* transformation

Genomic DNA extracted from the primary *A. nidulans* transformant AYY1 was used to transform electrocompetent *E. coli* to ampicillin resistance, indicating rescue of the transforming plasmid. One microliter genomic DNA was mixed with 40 µL ice-thawed electrocompetent XL-1 Blue then transferred to a prechilled Gene Pulser /*E. coli* pulser Cuvette (BIO-RAD laboratories, www.bio-rad.com). Electroporation was carried out by using a TransPorator (BTX, www.btxonline.com) at 2.5 kv for 5 milliseconds. Two hundred fifty microliter SOC solution was added to the cuvette immediately for recovery of the electroporated cells. Transformed cells were incubated at 37°C for 30 minutes, then diluted as appropriate to plate over the selective LB plus ampicillin media followed by incubation at 37°C overnight. An ampicillin resistant colony was isolated for extraction of plasmid containing the *hypB5* complementing DNA fragment, which was named pYY1.

2.2.1.7 Subcloning a *hypB5* complementing fragment from pYY1

The pYY1 plasmid was digested with a panel of restrictive endonucleases including *KpnI*, that are found in the pBluescript multiple cloning site (MCS). The pool of restrictive fragments of pYY1 (about 4 µg) was tested for complementation activity by co-transforming into ASK166 with 1 µg autonomous replication plasmid (ARp1, containing AMA1, *N. crassa pyr4* and Amp^R) (Gems *et al.* 1991). The pYY1 digested with *KpnI* was able to complement the *hypB5* restrictive phenotype, indicating that this endonuclease did not cleave within an essential region of the complementing DNA.

pYY1 (10 µg) was digested at 37°C for 2 hours with *KpnI* (2 U/µg DNA) in a final volume of 20 µL containing 1X restriction buffer. The *KpnI* digest was separated by electrophoresis in a 0.55% agarose gel prepared in TAE buffer. Electrophoresis used 80 volts for 2 hours until the tracker dye migrated to the end of the gel. The gel was stained with ethidium bromide for 10 minutes and washed in water for another 10 minutes. Restriction fragments were imaged by using a long wave UV light then cut from the gel. Purification of DNA was performed with the CONCERTTM Rapid Gel Extraction kit (Life Technologies, www.lifetech.com) according to the supplier's recommendations. Individual fragments were completely separated from each other as proven by a 1/10 volume of each purified fragment run on a 0.8% agarose gel. Co-transformation of ASK166 with ARp1 (1 µg) and individual *KpnI* fragments (about 1 µg) was used to identify the *hypB5* complementing fragment in pYY1.

2.2.1.8 Ligation of 5kb *KpnI* fragment with the pBCKS+ vector

In the vector pBCKS+, the MCS is part of the *LacZ* coding region which can generate functional β-galactosidase. A large fragment of foreign DNA inserted into the MCS of pBCKS+ will disrupt the production of β-galactosidase. IPTG, a potent inducer of the *Lac* operon, binds the *Lac* repressor, consequently inducing β-galactosidase synthesis. Colonies expressing active β-galactosidase are blue when grown on plates containing IPTG and X-Gal; while colonies without an active β-galactosidase are white on these plates (Sambrook and Russel, 2001).

pBCKS+ was digested with *Kpn*I coupled with Calf Intestinal Alkaline Phosphatase (CIP) treatment to remove the 5' phosphate residues from DNA and prevent self-ligation. Both vector DNA and purified 5 kb *Kpn*I fragment from pYY1 were extracted with phenol/chloroform, then by chloroform to prevent DNA degradation. Ligation used a 3:1 ratio of insert to vector, together with 4 μ L T4 ligase buffer and 1 μ L T4 ligase in a final volume of 20 μ L. The ligation reaction was incubated at 16°C overnight. The reaction product was ethanol precipitated, then resuspended in 10 μ L distilled water. XL-1 Blue cells were transformed with 2.5 μ L ligation mix suspended in water. Transformed cells were incubated on LB+chloramphenicol+IPTG+Xgal plates overnight to select for cells containing plasmid with the insertion. Plasmids were purified from white colonies, and then digested with *Kpn*I. The expected construct contained a 5 kb insertion band and the 3.4 kb pBCKS+ vector. This was named pYY2.

2.2.2 Microscopic observations

A. nidulans strains were grown on coverslips in liquid media with 100 μ g/mL ampicillin (Kaminskyj, 2001). Five milliliters of media containing 5×10^4 conidia/ml was poured into a 60 mm Petri dish containing a glass coverslip, and incubated at 28°C or 42°C for 18 hours. Conidia adhere to the coverslip during germination. For upshift experiments, cultures were incubated at 28°C for 18 hours, then 42°C for 6 hours. For downshift experiments, cultures were incubated at 42°C for 18 hours, then 28°C for 3 hours.

The coverslips with adhering germlings were fixed in 1X fixative (See Table 2.2) for 30 minutes. Coverslips were then rinsed with water, incubated 5 minutes with 10 μ g/mL Calcofluor (American Cyanamid, Wayne, NJ) to stain cell walls and 100 ng/mL Hoechst 33258 (Sigma, St. Louis) to stain nuclei. The coverslips were rinsed again twice with water then mounted in 5 μ L mounting solution (See Table 2.2) on a microscope slide. The slides were dried in an air flow hood for 30 minutes then sealed with nail polish. Germlings were examined using a Zeiss (Thornwood, NY) Axioplan microscope and photographed with a Sensys digital camera (Roper Scientific, www.inovis.com) controlled by Meta Vue software (Universal Imaging, www.imagem.com). Germling

features were measured with the Meta Vue software. Subapical cell length was measured in unbranched cells with complete septa perpendicular to the long axis of the hypha. The number of nuclei in subapical cells was counted in the same cells as used for measurement of subapical cell length. Hyphal widths were measured at the complete septa perpendicular to the long axis of the hypha. Fifty data points were collected for each parameter. ANOVA and T-test analyses used internet software (www.physics.csbsju.edu/stats/anova.html, www.physics.csbsju.edu/stats/t-test.html).

2.2.3 pYY2 DNA sequencing

2.2.3.1 Transposon-facilitated DNA sequencing

As a conventional strategy, for DNA fragments >1 kb, a primer walking and/or restrictive enzyme mediated subcloning is needed to sequence the whole length of the target. It is not practical to sequence both strands of a DNA fragment longer than about 800 bp from terminal priming sites. An efficient strategy is to create general priming sites by transposon insertional mutagenesis within the target DNA sequence (Strathman *et al.* 1991). Two criteria are notable 1) the transposon must insert into the target DNA sequence but not the plasmid vector, 2) the transposon insertion site must be mapped easily (Strathmann *et al.* 1991).

Tn1000 is a transposon of about 6 kb (Broom *et al.* 1995) that belongs to the $\gamma\delta$ family. $\gamma\delta$ is expressed on the F⁺ factor. By conjugating with a target sequence, an intermediate is formed named a cointegrand. The transient complex containing two copies of Tn1000 will be rapidly resolved by a site specific recombination system which is under the control of the transposon. As a result, both the F⁺ factor and the targeted plasmid will have a single copy of $\gamma\delta$. This transposon family is activated by mating and inserts randomly into the plasmid. Insertions into the plasmid backbone are likely to disrupt function and be lethal; hence, rescued plasmids are likely to have transpositions into the insert. The $\gamma\delta$ family members contain a 38-base-pair terminal inverted repeat sequence as their common feature. The reverse repeats are out-wards facing from the transposon, conveniently providing a primer “GDIR” binding site for mapping the insertions. The subterminal sequences adjacent to GDIR provide primer GD1 and GD2

binding sites introduced into the unknown DNA sequence to be utilized for further DNA sequencing. The cartoon in figure 2.2 provides a scheme for the Tn1000 transposon. GD1, GD2 and GDIR sequences are given in Table 2.3.

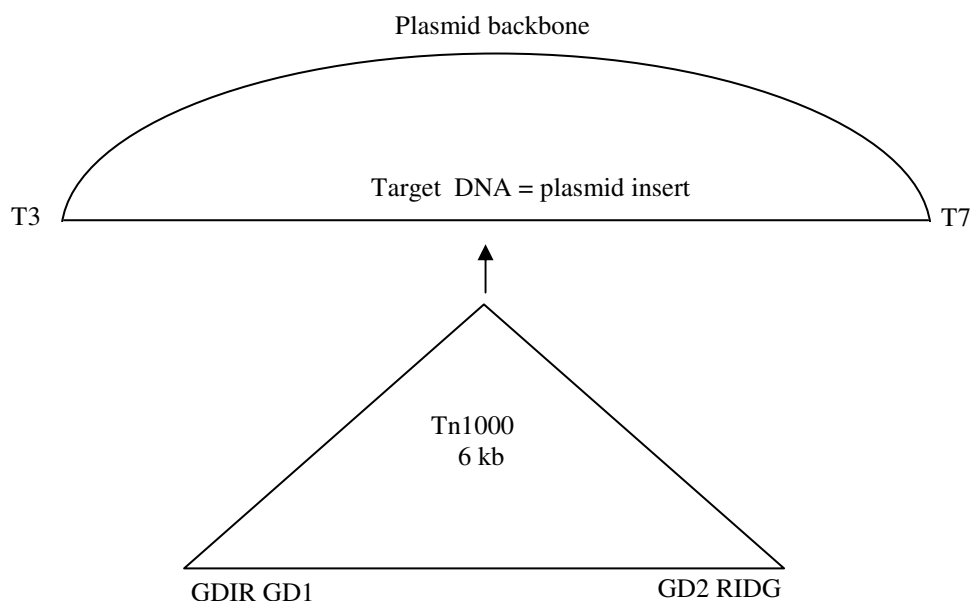


Fig. 2.2 The structure of Tn1000. At both ends, the 38-base reverse repeats can be used as GDIR primer binding sites. The subterminal sequences adjacent to GDIR can be used to provide GD1 or GD2 primer binding sites. Thus PCR with the primer pairs T3/GDIR and T7/GDIR can determine the insertional map, while GD1 and GD2 can be used for DNA sequencing of the “target” DNA, thus the full length of an unknown DNA sequence can be determined by using a panel of clones with different transposon inserts.

2.2.3.2 Mating of DH5 α and XL-1 Blue

DH5 α , the spontaneous rifampicin resistant strain, and XL-1 Blue containing pBluescript (pYY2, Tn1000, chlor^R) were grown overnight under appropriate selection. A single colony of each strain was inoculated in 10 mL LB liquid with the appropriate antibiotics for incubation at 37°C overnight with shaking at 250 rpm. Cells were pelleted and resuspended in 1 mL LB. Three hundred microliters of XL-1 Blue suspension was gently mixed with 200 μ L DH5 α suspension and incubated at 37°C for 60 minutes without shaking. One milliliter prewarmed (37°C) LB was added to the mating mix for three hours more incubation at 37°C without shaking. The mating culture was concentrated to 200 μ L LB and plated on LB agar containing both rifampicin at 100 μ g/mL and chloramphenicol at 100 μ g/mL. The plate was incubated overnight at 37°C. This plate produced colonies that contained the putative Tn1000 insertion.

2.2.3.3 Mapping Tn1000 Insertions

PCR was used to map the position of the Tn1000 insertions in the pYY2. For this purpose, three primers were used. One (GDIR) matches the inverted repeats at both ends of Tn1000. The other two are general primers (T3 and T7) flanking the MCS of pBluescript. Two PCR amplifications with T3/GDIR and T7/GDIR as primer pairs were used to determine each insertion point. Mapping was performed on 100 independent inserts from the mating experiment. PCR was carried out in a 25 μ L reaction volume containing 1X TNK 50 buffer (See Table 2.2), 0.2 mM dNTPs, 0.2 μ M each primer, 2.5 U *Taq* DNA polymerase and 0.5 μ L overnight Tn1000 insertion culture as template. The PCR condition for T3/GDIR was 94°C for 3 minutes, then 30 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes, then 15 minutes at 72°C for extending. For T7/GDIR the annealing temperature was 62°C instead. Ten microliter of the PCR reaction was subsequently analyzed by agarose gel electrophoresis. The primers GD1/GD2 which are complementary to Tn1000 sequence adjacent to the terminal inverted repeats were used for pYY2 sequencing. PCR amplifications employing T3/GD1, T3/GD2, T7/GD1 and T7/GD2 as primers were performed to determine the

directions of GD1/GD2 in terms of each single insertion. The annealing temperature for GD1 is 58°C, whereas it is 53°C for GD2.

2.2.3.4 pYY2 DNA sequencing

The Tn1000 transposon was introduced into pYY2 by a transposition event during bacterial mating. Sequences at the transposon ends were used as primer binding sites for sequencing. Cycle sequencing (Hillis *et al.* 1996) is a modification of the traditional Sanger sequencing method (Sanger *et al.* 1977). Cycle sequencing employs a thermostable DNA polymerase which can be heated to 95°C and still retain activity. The advantage of using such a polymerase is that the sequencing reaction can be repeated in the same tube by heating the mixture to denature the DNA and then allowing it to cool to anneal the primers and polymerize new strands. Thus, less template DNA is needed than for conventional sequencing reactions. Because there is a low concentration of dideoxy nucleotide in the reaction mixture, the newly synthesized DNA strands vary in length. Based on their size difference, these strands can be separated electrophoretically in polyacrylamide gels. Modern sequencing procedures employ fluorescent chemical markers that are detected as the DNA fragments pass a stationary laser. The DNA sequence is recorded as a chromatogram where each "peak" corresponds to a single nucleotide. The chromatogram is recorded by computer and translated into a DNA sequence. In general, sequencing strategies should include both strands of the DNA fragments, as complementary strand confirmation leads to higher accuracy.

PRISM Ready Reaction Mix, purchased from Perkin-Elmer Applied Biosystems Division, contains the buffers, nucleotides, fluorescent-labeled dideoxy terminators and enzymes needed for the sequencing reactions in a single tube. Since each type of dideoxy terminator (ddATP, ddGTP, ddCTP and ddTTP) is tagged with a different colored fluorescent dye, all four terminators could be mixed in a single reaction and loaded onto a single lane on the gel. This reaction uses thermostable DNA polymerase to allow repeated cycles of sequencing reactions, to enhance the signal from small amounts of template. The thermostable polymerase has the additional benefit of reducing effects of

DNA secondary structure on the reaction (by using higher temperatures to melt the template). Ideally the reaction will allow up to 700 base pairs to be readable.

The cycle sequencing reagent was mixed in a final volume of 10 µL, including 2.5 µL Sequencing Buffer (Perkin Elmer, www.perkinelmer.com), 2 µL Big Dye (Perkin Elmer), 16 pM primer and 0.4 µg plasmid DNA. The reaction was performed in the PTC-200 DNA EngineTM Peltier Thermal cycler (MJ Research, Inc., www.mjr.com/index.html). The PCR program was 80°C 4 minutes; 24 cycles of 96°C for 10 seconds, 50°C for 5 minutes and 60°C for 4 minutes. The sequencing products need to be purified to remove the excess terminators which otherwise will result in ambiguous chromatograms. For each reaction, the total PCR product was transferred into a 200 µL microfuge tube to mix with 1 µL 3 M pH 5.2 sodium acetate and 25 µL -20°C 95% ethanol then incubated on ice for 10 minutes. DNA was precipitated at 13000 rpm for 15 minutes. After washing with 70% ethanol, the DNA was dried in a vacuum centrifuge for 15 minutes. The purified DNA was mixed with 3.2 µL loading buffer TSR (Applied Biosystems, <http://home.appliedbiosystems.com>) and denatured at 94°C for 3 minutes, then immediately transferred to ice to prevent reannealing of the single strand DNA. The reactions were loading in an ABI Prism 377/310 DNA sequencer (Applied Biosystems) in the Biology Department at the University of Saskatchewan. On average, about 500-700 base pairs were readable.

2.2.3.5 Assembly of pYY2 sequence

The full length of pYY2 was assembled using Sequencer 3.1.1 (Gene Codes Corporation, www.genecodes.com). The parameters for contig assembly were 85% minimum match and at least 20 base pairs overlapped. Every single base pair was sequenced in both directions to ensure the accuracy. Finally, the full length of pYY2 sequence was assembled into one contig.

2.2.4 Transposon-arrayed pYY2 disruption

Transposon-arrayed gene disruption is an approach for understanding gene function by insertional mutagenesis (Hamer *et al.* 2001). Tn1000 is about 6kb in size (Broom *et al.*

1995), large enough to disrupt gene function when inserted into an essential region of a target gene. A collection of Tn1000 insertions in a gene of interest can be used to identify regions that are essential for function. A set of Tn1000 insertions into pYY2 about 500 base pairs away from each other were chosen to analyze the essential part of pYY2. Cotransformation of ASK166 with ARp1 (1 µg) and individual pYY2 plasmids with Tn1000 inserted at distinct regions (about 4 µg) was as described in 2.2.1.3. Transposon events that did not prevent pYY2 from complementing the *hypB5* restrictive phenotype were interpreted as not having disrupted an essential part of the gene.

2.2.5 Analysis of pYY2 sequence

pYY2 sequence was analyzed by using the internet program including: BLAST(www.ncbi.nlm.nih.gov/blast), ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html), Prosite (ca.expasy.org/cgi-bin/scanprosite), Clustal W(www.ebi.ac.uk/clustalw/), Expasy (ca.expasy.org/cgi-bin/protparam) or the software MODELLER (Šali and Blundell, 1993).

2.2.6 Mating *Aspergillus nidulans* strains

Fresh conidia of two strains having different spore colors and nutritional markers were inoculated in 2 mL CM liquid medium with 100 µg/mL ampicillin in a 24-well tissue culture dish. Equal numbers of conidia from both parental strains were used for mating. Complementary auxotrophies of the parental strains are required for selection of heterokaryons when transformed to MM media, increasing the chances of finding an outcrossed cleistothecium. Different colors of the parental strains will make it easier to identify outcrossed cleistothecia. The liquid culture was grown at 28°C for two days. The hyphal mat which formed over the liquid medium was transferred to MM agar plates and incubated at 28°C for another 2 days, then were sealed with parafilm and stored in the dark for 2-3 weeks for the cleistothecia to mature. Outcrossed cleistothecia are frequently found on the hyphal mat near conidia with both parental colors. Hülle cells covering the cleistothecia were cleaned with 4% water agar containing 1% diatomaceous earth (Kaminskyj and Hamer, 1998). The cleaned, putative outcrossed, cleistothecia were

transferred to an Eppendorf tube with 200 μ L sterile distilled water, crushed and vortexed to release the ascospores. The ascospore suspension was plated over CM supplemented for all auxotrophies to allow the formation of pigmented conidia. Outcrossed cleistothecia contained progeny of both parental colors. Non-allelic genes will give rise to wild type progeny under selective conditions.

2.2.7 Identification of *hypB5* strain Sec7 domain lesion

For *hypB5* Sec7 domain lesion identification, the putative mutation region was amplified by PCR. PCR primer design followed a set of rules to get the optimal PCR result: 1) primers should be 17-28 bases in length, 2) base composition should be 50-60% GC, 3) primers should 3'-end with G or C to increase priming efficiency, but avoid three or more Cs orGs which may promote mispriming at G or C-rich sequences (because of stability of annealing), 4) primer dimer should be prevented, 5) primer self-complementarity such as the formation of a hairpin should be avoided. The distance between primer binding sites was below 500 base pairs to facilitate the sequencing limitation. Primers were designed with the internet software (<http://alces.med.umn.edu/rawprimer.html>).

In order to amplify a specific region of the pYY2 sequence from a *hypB5* strain, ASK166 genomic DNA was prepared as described in 2.2.1.3. PCR reactions were performed in 25 μ L final volume with 2.5 μ L 10 X PCR buffer #8 (See Table 2.2), 0.2 mM dNTPs, 0.2 μ M primer of each, 2.5 U Taq DNA polymerase and 2 μ L ASK166 genomic DNA. The PCR condition was first 5 cycles of 95°C for 1 minute, 49°C for 1 minute and 72°C for 1 minute; 25 cycles of 95°C for 1 minute, 52°C for 1 minute and 72°C for 1 minute; final extension of 72°C for 5 minutes. PCR products were purified by adding 50 μ L PEG and incubating at 37°C for 15 minutes then centrifuged at 15000 rpm for 15 minutes. The DNA pellet was washed with cold 80% ethanol and air dried. The purified DNA was resuspended in 25 μ L TE and used as sequencing template as described in 2.2.3.4. The sequence data of *hypB5* was used to compare to wild type to find the putative mutated site.

2.2.8 pYY2 cDNA

2.2.8.1 *Aspergillus nidulans* total RNA preparation

All materials used in preparation of RNA were RNase free.

Freshly harvested A28 spores were grown in 50 mL CM liquid with 100 µg/µL ampicillin at 28°C for 16 hours. The mycelia were dried and ground to fine powder in liquid nitrogen, then transferred to a 50 ml Falcon tube. Fifteen milliliters of RNA extraction buffer was added to the tube followed by vortexing to disperse the mycelia, then 13 mL phenol/chloroform was added to the same tube and mixed by shaking. After centrifuging at 2000 rpm for 15 minutes at 4°C, the top phase was moved to a new tube. Extractions were repeated for four times, with the last extraction using chloroform only. The final supernatant was transferred to a 30 mL Corex tube for precipitation of RNA/DNA mix by adding 1/12 volume of 4 M sodium acetate and 0.8 volume of isopropanol and incubation at -20°C over night. RNA/DNA mix was pelleted by centrifuging at 8000 rpm for 30 minutes at 4°C. The pellet was resuspended in 5 mL water. RNA was precipitated by adding 5 mL 4 M LiCl followed by incubation on ice for 2 hours, then centrifuging at 4°C 8000 rpm for 30 minutes. RNA was washed with 70% ethanol twice then dissolved in 100 µL water and kept at – 80°C. The concentration of RNA was measured by spectrophotometer and gel electrophoresis.

2.2.8.2 *Aspergillus nidulans* mRNA preparation

mRNA extraction was performed by using PolyATtract® mRNA isolation systems (Promega, www.promega.com). About 2 mg total RNA in 500 µL water was heated at 65°C for 10 minutes. Three microliter of Biotinylated-Oligo (dt) probe coupled with 13 µL 20 X SSC was mixed with the RNA and incubated at room temperature for 10 minutes. The Streptavidin-Paramagnetic particles (SA-PMPs) were resuspended by flicking the bottom of the tube then captured by placing the tube in the magnetic stand until the SA-PMPs were captured. The supernatant was discarded without disturbing the SA-PMPs. The particles were washed three times by using 0.3 mL 0.5 X SSC and resuspended in 0.1 mL 0.5 X SSC. RNA, after the annealing reaction, was transferred to mix with the washed SA-PMPs and incubated at room temperature for 10 minutes. The

SA-PMPs were washed four times with 0.1 X SSC and resuspended in a final volume of 0.25 mL water. mRNA was concentrated by adding 0.1 volume 3 M sodium acetate (pH 5.2) and 1.0 volume isopropanol and incubation at -20°C over night. mRNA was pelleted by centrifuging at 12000 rpm for 10 minutes at 4°C. The pellet was washed once with 75 % ethanol, air dried, and resuspended in 20µL water. mRNA was stored at -80°C.

2.2.8.3 *Aspergillus nidulans* cDNA preparation

In a final volume of 20 µL, two microliter of mRNA was mixed with 52 pmol oligo-dT16 primer and 9 µL water for heating at 70°C for 10 minutes. The tube was chilled on ice immediately to prevent reannealing. Four microliters of 5 X First-strand reaction buffer (Invitrogen, www.invitrogen.com), 0.2 mM DTT (Invitrogen), 10 µM dNTPs and 10 U RNAsin was added into the same tube for 3 minutes incubation at room temperature. Finally, 200 U reverse transcriptase (Invitrogen) was added into the tube for an hour incubation at 42°C.

2.2.8.4 cDNA PCR

PCR reaction was performed in a final volume of 25 µL with 2.5 µL 10X PCR reaction buffer, 0.2 mM dNTPs, 0.2 uM primer of each, 2.5 U Taq DNA polymerase and 0.5 µL 1:10 diluted A28 cDNA. PCR reactions were also carried out by using *pfx* DNA polymerase (Invitrogen) according to the manufacture' s recommendation. Various primers combinations were tested to detect the potential cDNA coding region.

3. RESULTS

3.1 Cloning of *Aspergillus nidulans* Sec7 coding gene

3.1.1 *Aspergillus nidulans* transformation

The *A. nidulans* pRG3-AMA1 genomic library (Oshero *et al.* 2000a, b) was established by ligating partially *Sau3AI*-digested *A. nidulans* genomic DNA into a *Bam*HI digested vector which contains: 1) a sequence for autonomous maintenance in *Aspergillus* (AMA1), 2) the coding region of *Neurospora crassa pyr4* gene, 3) the ampicillin resistance marker (Aleksenko and Clutterbuck, 1997). AMA1 is the only *A. nidulans* genomic region mediating an autonomous plasmid replication. AMA1-bearing plasmids are able to enhance fungal transformation frequency up to 2000-fold due to their exclusive location in the nuclei and average 10-20 copies per nucleus. In addition, fifty-six repeats of the *SpeI* motif (ACTAGT) embedded in the AMA1 sequence may play a role in increasing the transformation frequency (Aleksenko and Clutterbuck, 1997). The autonomous replication capacity and ampicillin resistance allow the rescue of the plasmid from transformants to *E. coli* and subsequent cloning. As a selectable marker, *Neurospora crassa pyr4* ensures that *Aspergillus* transformants which uptake the plasmid can grow on the media without pyrimidines, which reduces background.

ASK166 (*pyrG89*; *wA2*; *hypB5*; *veA1*) was used as the recipient strain for cloning the *hypB5* complementing sequence by phenotype complementation. Only transformed protoplasts that received a plasmid bearing *hypB* wild type copy or an extragenic suppressor of the *hypB5* mutation (as well as *pyr4*) should resemble wild type at 42°C on medium lacking pyrimidines. In contrast, strains containing the *hypB5* allele germinate and grow slowly at 42°C with abnormal morphology. Protoplasts were allowed to reestablish cell walls at 28°C before applying selection pressure at 42°C. Improved transformant survival was observed using this temperature regime in preliminary studies. After the first round of transformation (Fig. 3.1), about 15 strains showing wild type phenotype at 42°C were isolated and one of them was named AYY1. The controls

showed that there was no contamination from wild type strains since ASK166 has white spores whereas wild type spores are green. Only restrictive growth was observed of the cells whose *pyr* auxotrophy was complemented but not the *hypB5* defective phenotype. The colonies (Fig. 3.2) and cellular (Fig. 3.3) morphologies showed that unlike the parental strain ASK166, the transformed AYY1 resembled wild type at the restrictive temperature (Fig. 3.2 and 3.3). Cellular morphometry of wild type, transformed AYY1 and *hypB5* strains are given in Table 3.1.

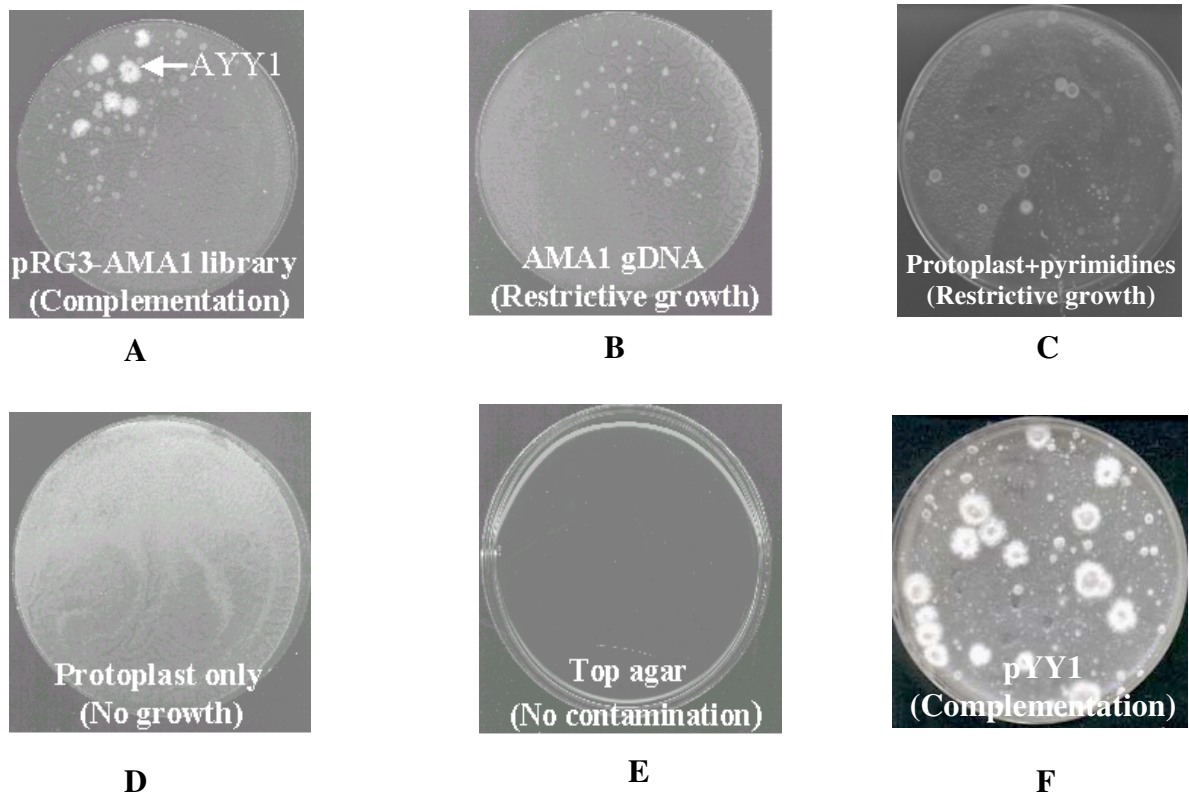


Fig 3.1 Cloning *Aspergillus nidulans* *hypB* by complementing the *hypB5* phenotype at 42°C. (A) Strain ASK166 (*pyrG89*; *wA2*; *hypB5*; *veA1*) was transformed with the pRG3-AMA1 *A. nidulans* genomic library, leading to the growth of wild type colonies. (B) Transformation with the AMA1-*pyr4* vector backbone alone led to *hypB5* restrictive growth on medium lacking pyrimidines. Controls: ASK166 protoplasts grew with the restrictive phenotype on medium containing pyrimidines (C), but there was no growth on medium lacking pyrimidines (D). (E) The top agar used for plating transformants was not contaminated with wild type spores. (F) The complementing plasmid was rescued from transformant AYY1 (indicated in A) and used to transform *E. coli* XL1-Blue to ampicillin resistance, creating pYY1. pYY1 complemented the *hypB5* restrictive phenotype when transformed to ASK166.

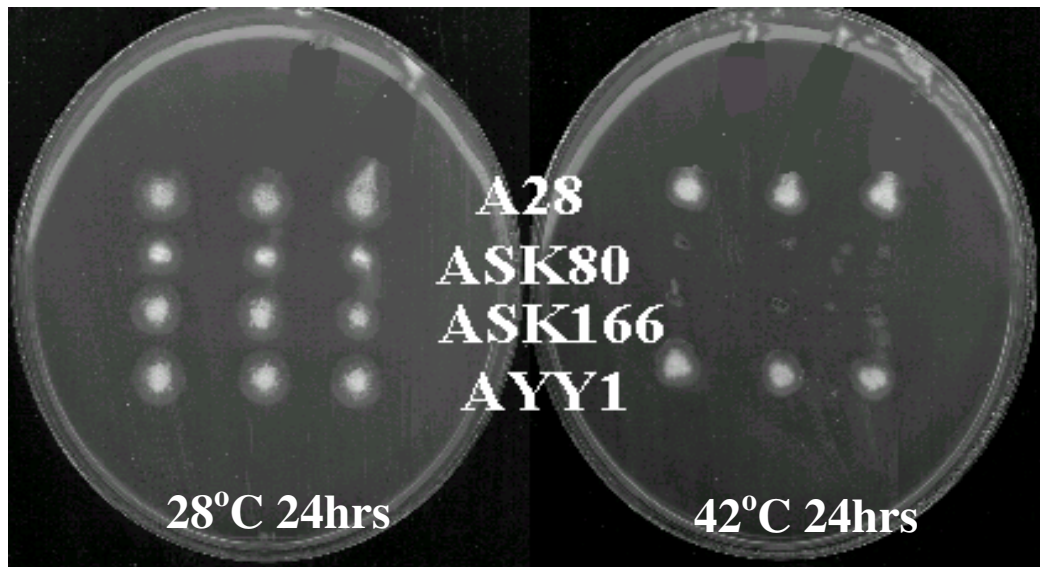


Fig 3.2 The colony morphologies of wild type (A28), *hypB5* (ASK80 and ASK166) and AYY1 (*hypB5* strain bearing *hypB5* complementing DNA) grown at 28°C and 42°C for 24 hours. The growth of *hypB5* mutants was inhibited at 42°C, but the AYY1 strain that has a DNA complementing *hypB5* shows wild type growth similar to A28 at 42°C.

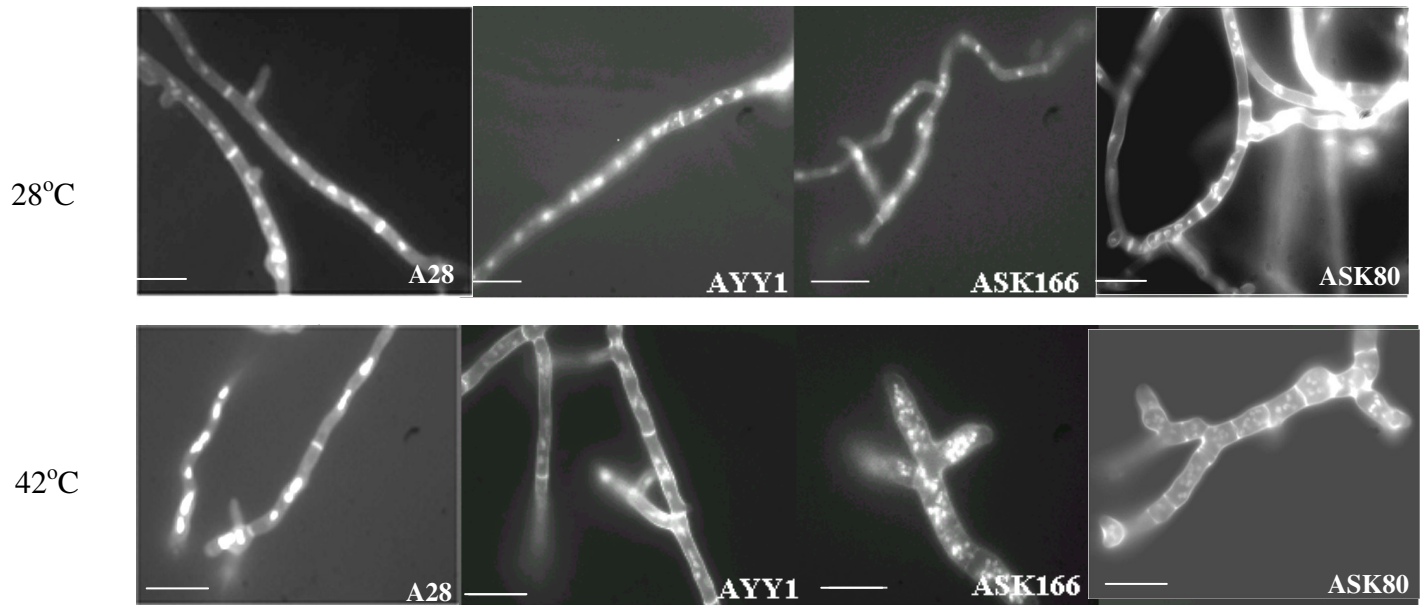


Fig. 3.3 Cellular morphologies of wild type (A28), AYY1 and *hypB5* (ASK166 and ASK80). Only the transformed AYY1 strain bearing *hypB5* complementing DNA resembled wild type parent at 42°C. Cells were incubated at 28°C or 42°C followed by Calcofluor staining for cell walls and Hoechst 33258 for nuclei. Bar 10 μm.

3.1.2 Microscopic observations

A. nidulans wild type strains produce narrow cylindrical hyphae at both permissive temperature (28°C) and restrictive temperature (42°C). At 28°C, *hypB5* mutant strains resemble wild type, but at 42°C they have shorter subapical cells, wider hyphae and more nuclei than wild type. Unlike the parental strain ASK166, and another *hypB5* strain ASK80, the transformed AYY1 colonies grew like wild type at both permissive and restrictive temperatures (Fig. 3.2 and 3.3). The cellular morphometric characteristics of wild type, transformed AYY1 and *hypB5* mutant strains are presented in Table 3.1. Statistical analysis (ANOVA and t-test) showed that the cellular morphologies of the transformed AYY1 strain grown at 42°C were much more like wild type than *hypB5* mutants after transformation with the *hypB5* complementing DNA, but not identical in all respects. Basal cells of wild type strains are about 40 µm long and 3 µm wide, when measured after 24 hours growth in regions of the hyphae far from the spore. Each subapical cell contains about 3-4 nuclei. Wild type hyphae have longer and wider basal cells, but fewer nuclei per subapical cell than AYY1 at both temperatures. Although, the statistical analysis indicated that AYY1 possesses smaller cellular volume but harbors more nuclei than wild type will be further discussed in 4.3.

Table 3.1 Characteristics of basal cells of *A. nidulans* strains

	A28	AYY1	<i>hypB5</i> (ASK166)	<i>hypB5</i> (ASK80)
Subapical cell length				
(μm) \pm SE				
28°C	23.3 \pm 7.9 ^a	19.8 \pm 6.5 ^a	18.6 \pm 5.7 ^a	20.4 \pm 7.9 ^a
42°C	19.2 \pm 6.2 ^a	13.7 \pm 5.1 ^b	6.2 \pm 2.4 ^c	6.0 \pm 2.3 ^c
Hyphal width				
(μm) \pm SE				
28°C	3.7 \pm 1.1 ^a	2.6 \pm 0.3 ^b	2.3 \pm 0.4 ^b	2.5 \pm 0.3 ^b
42°C	3.7 \pm 1.5 ^a	2.8 \pm 0.3 ^b	5.5 \pm 0.7 ^c	5.7 \pm 1.1 ^c
Number of nuclei per				
subapical cell \pm SE				
28°C	2.5 \pm 0.4 ^a	4.4 \pm 1.0 ^b	3.2 \pm 1.1 ^{ab}	4.1 \pm 1.6 ^b
42°C	2.3 \pm 0.2 ^a	4.6 \pm 1.9 ^b	6.5 \pm 3.1 ^c	6.7 \pm 3.5 ^c

Germling features were measured as described above. Fifty measurements were collected for each parameter. Analysis was by ANOVA ($p < 0.05$) (www.physics.csbsju.edu/stats/anova.html) and t-test (<http://www.physics.csbsju.edu/stats/t-test.html>). Within each feature (basal cell length, width, number of nuclei) values that were statistically different ($p < 0.05$) are followed by a different letter. For each feature there were significant differences between wild type and *hypB5* strains. AYY1 has shorter basal cells than wild type, but significantly longer than *hypB5* strains. The cellular morphologies of the transformed AYY1 strain grown at 42°C were much more like wild type than *hypB5* mutants, particularly for basal cell length and hyphal width. However not all cellular features of AYY1 were similar to wild type.

3.1.3 Rescue of plasmid pYY1

In general, transformation is the introduction of foreign DNA into cells. Cells that are transformed will display subsequent inheritance and expression of that DNA under appropriate conditions. The shuttle vector that can be used to transform both *A. nidulans* and *E. coli* simplifies rescue of the plasmid of interest into *E. coli* for amplification. Genomic DNA was extracted from *A. nidulans* strain AYY1 and used to transform *E. coli* XL-1 Blue to ampicillin resistance. The *hypB5* complementing plasmid pYY1 was isolated by the method described in 2.2.1.5. To confirm the *hypB5* phenotype complementing activity of pYY1, plasmid DNA of pYY1 was prepared using alkaline lysis (Sambrook and Russel, 2001) and used for the second round of transformation. pYY1 was verified to complement *hypB5* defects (Fig. 3.1 panel f).

3.1.4 Construction of plasmid pYY2

pYY1 was digested with a panel of restrictive endonucleases including *KpnI*, *BamHI* and *SphI* to subclone the *hypB5* to the smallest complementing region. If the restrictive cutting site does not fall within an essential part of the complementing DNA, then the digestion product should have the same complementation activity as pYY1. This takes advantage of the fact that *A. nidulans* can be transformed by linear DNA fragments given cotransformation with an AMA1-*pyr4* helper plasmid ARp1. The complementation activity was maintained in the *KpnI* digestion products. *KpnI* digestion of pYY1 produced five fragments: 12 kb, 7 kb, 5 kb, 1.2 kb and 0.65 kb. To determine which one conferred the phenotype rescue activity, these fragments were purified by electrophoresis, then co-transformed with ARp1 to identify the one that could complement ASK166 defective growth at 42°C. The 5 kb *KpnI* fragment was found to have *hypB5* complementation activity (Fig. 3.4), was cloned into pBCKS+ and named pYY2.

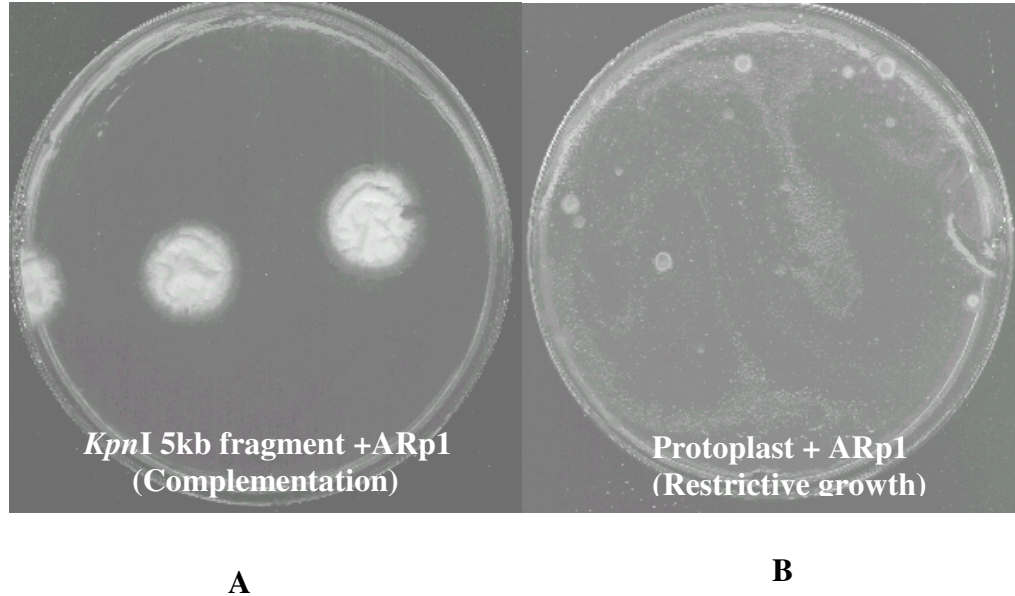


Fig. 3.4 Subcloning *hypB5* complementation fragment. Strain ASK166 (*pyrG89*; *wA2*; *hypB5*; *veA1*) was transformed with *KpnI* restriction fragments from pYY1. A 5 kb *KpnI* fragment was identified to have *hypB5* complementation activity after cotransformation of ASK166 with ARp1 (panel A). Control: ASK166 protoplasts after transformation with ARp1 showed only the restrictive phenotype on medium without pyrimidines at 42°C (panel B).

The unique *KpnI* cutting site in the MCS of pBCKS+ was used to ligate the 5 kb fragment generated by *KpnI* digestion of pYY1. Under the ligation condition, the 5 kb fragment could be inserted into the linearized empty vector to produce a plasmid which should be 8.4 kb. Transformation of this plasmid into XL-1 Blue should create white bacterial colonies which are able to grow on the selective LB+chloramphenicol+IPTG+X-Gal medium. Meanwhile, if competent cells only received the self-ligated vector, they should only show blue color on the same medium. To verify the potential correct insertion, a white colony was picked up from the selective medium for plasmid DNA extraction followed by *KpnI* digestion. *KpnI* digested plasmid DNA extracted from this colony after amplification and purification showed the expected 5 kb and 3.4 kb bands after electrophoresis. This plasmid was named pYY2. pYY2 was used for transposon facilitated DNA sequencing and Tn1000 disruption analysis.

3.2 Transposon-facilitated DNA sequencing

3.2.1 Mating DH5 α and XL-1 Blue and mapping Tn1000 insertions

By mating DH5 α (spontaneous rif^R mutant) with XL-1 Blue (pBluescript with insert pYY2, Tn1000, chlor^R), the transposon was activated to express the insertional activity. Only colonies surviving on LB+rif+chlor medium had one copy of Tn1000 inserted into pYY2. Because the insertion position was random, mapping the insertions was required for efficient DNA sequencing. Typically there is only one insertion per plasmid, whose position can be mapped by PCR. The primer pairs T3/GDIR and T7/GDIR were employed in the mapping of the insertion sites. Two groups of independent PCR reactions were used to map the Tn1000 insertions and show that the expected total size of the two PCR products was approximately 5 kb (Fig. 3.5). Eleven out of 100 insertions screened were chosen for having an even distribution along the 5 kb fragment (Fig. 3.5).

Since the GD1 and GD2 binding sites are adjacent to GDIR, either of them could face either T3 or T7. In order to clarify the sequencing direction, by using GD1 or GD2 as the sequencing primer, four PCR reactions were set up by using the combination of T3/T7 and GD1/GD2 as primers. It was expected that only two out of four reactions would generate PCR products, since nothing would be detected by gel electrophoresis

when using primers of the same direction. The size of the PCR products was expected to be approximately the same as that of using T3/GDIR or T7/GDIR. All of the eleven chosen insertions showed exactly what was expected (Table. 3.2). With the primer T3/T7 for sequencing ends of the pYY2 inserts and GD1/GD2 for the central area, both strands of pYY2 were completely sequenced. The information of the complementary sequence data ensured the accuracy.

Table 3.2 GD1 or GD2 directions of the eleven chosen Tn1000 insertions.

	#89 T3 /T7	#84 T3 /T7	#28 T3 /T7	#51 T3 /T7	#15 T3 /T7	#24 T3 /T7	#44 T3 /T7	#17 T3 /T7	#88 T3 /T7	#12 T3 /T7	#27 T3 /T7
GD1	+/-	-/+	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	-/+
GD2	-/+	+/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	+/-

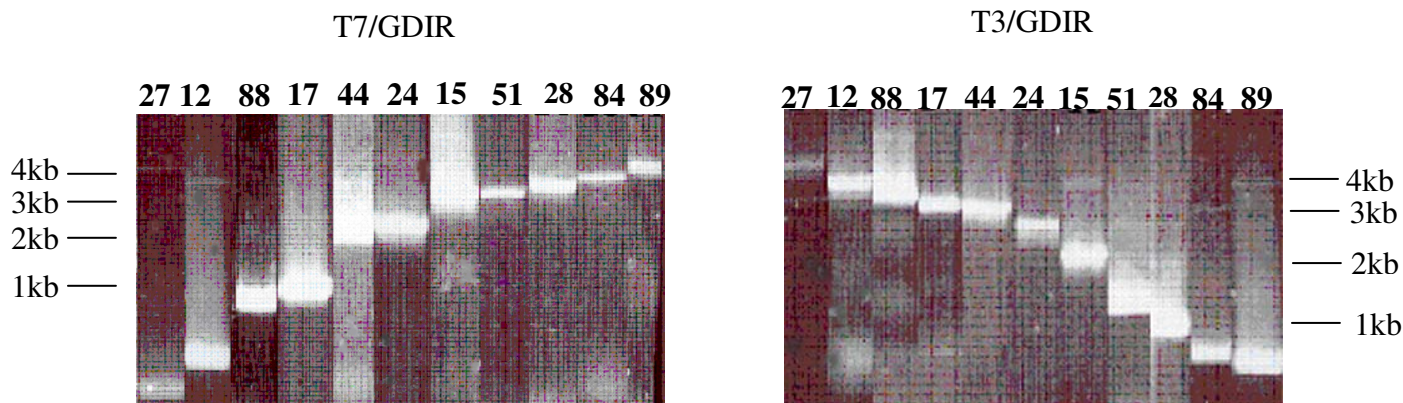


Fig. 3.5 A PCR using T7/GDIR and T3/GDIR primer set to map the positions of Tn1000 insertions into pYY2. The sum of the two PCR products for each clone was about 5 kb. This selection of clones has Tn1000 inserted into the pYY2 with approximately even spacing suitable for sequencing. The clone numbers are on the top of the panels.

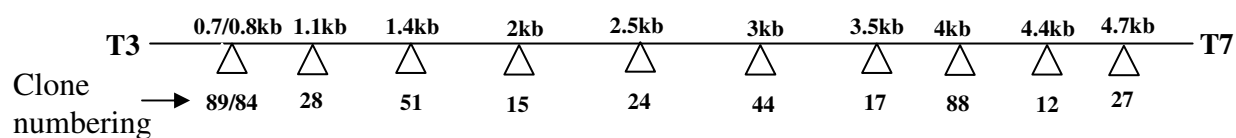


Fig. 3.5 B Eleven clones with Tn1000 insertions used for sequencing pYY2. The clone numbers are underneath each insertion. Distance (kb) starts from T3.

3.2.2 Assembly of pYY2 sequence

Using Sequencer 3.1.1, the individual DNA sequences from the transposon inserts were assembled into one contig. Overlaps were about 200 base pairs between readable sequences. The *KpnI* recognition sites were identified at each end of the pYY2 fragment. The full length of the 5 kb fragment was assembled into one contig (Fig. 3.6) completing pYY2 sequencing (Fig. 3.7). pYY2 sequence data have been deposited in GenBank under accession AY261780.

pYY2 sequence

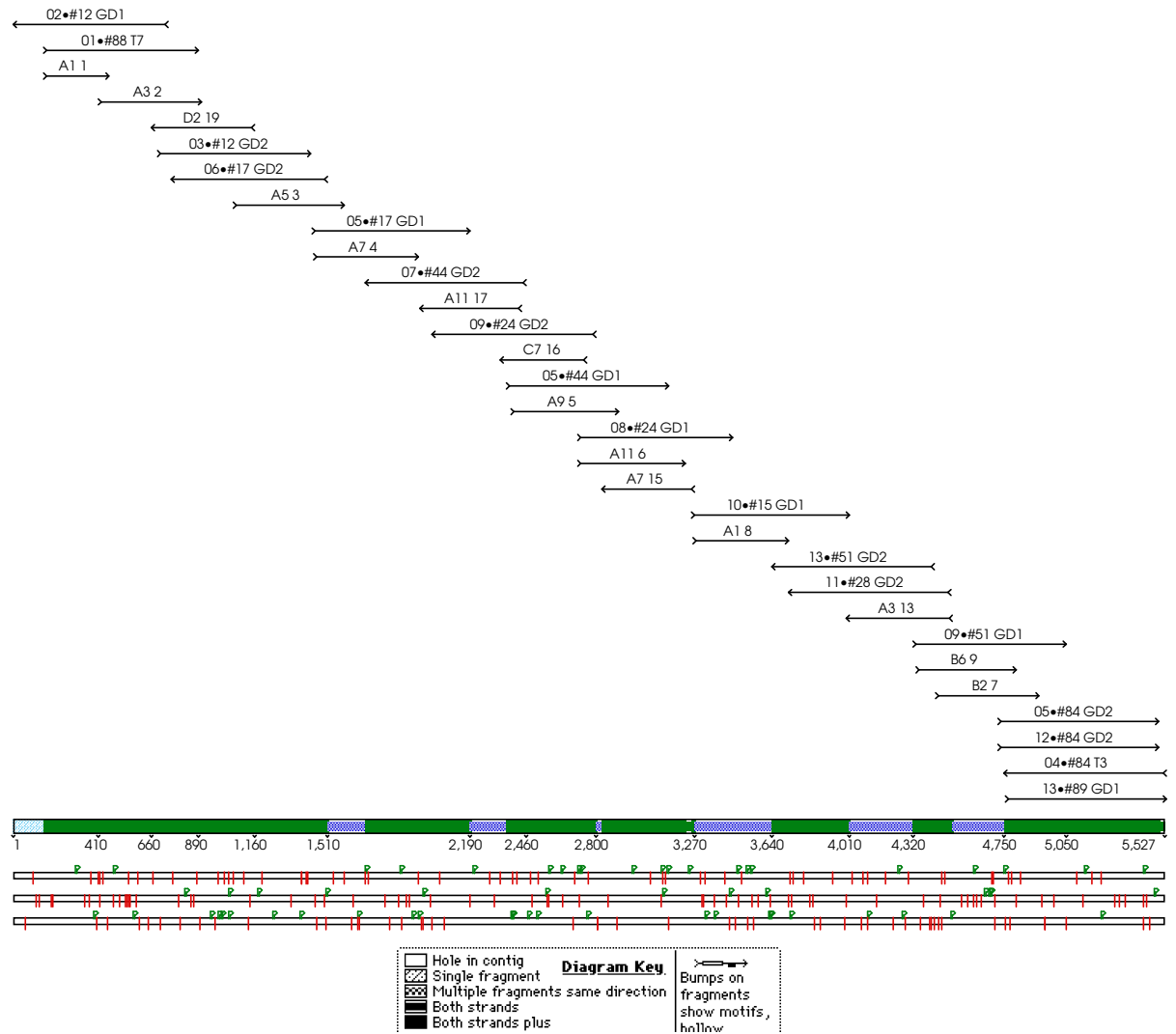


Fig. 3.6 The pYY2 contig assembly map from Sequencer 3.1.1.

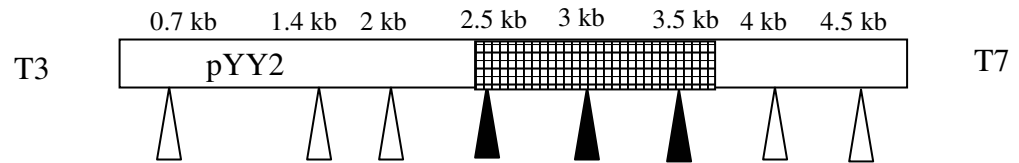
(KpnI) T3										
GGTACC CCCA	TTACTCCAGA	CGGAGTCTTG	CATAATGGCG	GAAGCTGAGA	ACGACCCTAC	GAACGAGCTC	AACCAAAACAT	80		
CAGTTACCCA	GGCAGACAAAG	CAGAATGGGG	TTGATGTTGC	AACAGAACCG	CACGCCCCGG	AGGTAGTCGC	GGAGTCAAAA	160		
CCAGCATTAA	CCGATGAATC	AGAACGTCAG	GAGATCCCAA	CAATTAAAGA	GAATGAGGAC	ACAATGGCGA	ACAACCGATT	240		
GAATGACAGC	AAGAATAACC	TGCCTCATGA	GCCCTCCGTA	ACGTCTCCGG	ATACTACCA	TGATTCAAAC	GAGCCTACCG	320		
ACGAACCAGA	ACAACCAAT	ACAGAAGGTG	ATCAACTCGA	AACTCTGCAG	CAAGATCAGC	CTCCTGCTTC	GGACGAACAA	400		
CTGAATGAAG	CTCCAGATGC	GCCGTCAACT	CGCGATGAGC	AGCTTGCCCA	GGATATGCGA	CAGCGATCGG	ACTCGCGATC	480		
GACTACCGCA	AGCTTCGCTA	CCAATCGGTC	CTCGGTAGTC	AGCTCTACTG	TTTTTATTGT	GACAGCTCTG	GATGCAATTG	560		
GTGCGTCGCG	GGAAGCTCGG	AAGAGCAAAG	AGCTGGAGGA	CGCTGTAAAG	AATGCGCTCG	CCAATGTCAA	GCAGTCGGAC	640		
CGCCAACCTA	TCGACCCGGA	GATACTGTTT	TACCCTTTAT	TACTCGCAAG	CAGGACTCTA	AGCATTCCCT	TGCAAGTAAC	720		
GGCTCTCGAT	TGCATCGGCA	AGCTAATCAC	CTACTCTTAT	TTTGCAATTC	CATCGGCGCA	AGAAGCCAAG	CCATCGGAGG	800		
CAGATGCTAC	GGCAGAGCAG	CCTCCTCTTA	TAGAGCGAGC	CATTGACGCT	ATATGCGATT	GCTTCGAAAA	TGAAGCTACG	880		
CCAATCGAGA	TCCAACAACA	GATAATCAAA	TCCCTTCTGG	CTGCAGTATT	GAACGACAAA	ATTGTGGTGC	ATGGAGCAGG	960		
TCTTCTGAAA	GCTGTTCCGC	AAATCTACAA	CATGTTTATT	TACTCAAAGT	CCAGTCAGAA	CCAACAAATC	GCTCAAGGAT	1040		
CCCTCACACA	AATGGTCAGC	ACGGTCTTTG	ACAGACTCGG	CGTTAGACTT	GATCTACGGG	AATTGCGCAT	CCGTGAAGGC	1120		
GAAAAGGCGC	AGGCGGGGTC	TTCGGAAAGT	GTGACTGTTG	AGCCTGTCTG	TAGCCCGCCA	TCTGCTGAGG	ACGATCAGGC	1200		
ATCTGATGTT	GCTTCTGTAG	CAGCGGATCA	ACCGGTCTCC	AAGGAGCCAA	CGGAAAAGCT	CACCTTTGGAG	AGCTTCGAAT	1280		
CGAACAAAGA	TGTTACAACA	GTCATGATA	ACGTACCAAC	AATGGTCAAC	CGCGCCAACA	TTAATCAGAA	AAGAAGCGCAG	1360		
TCTTACTACG	GCACCTTCCTC	GGAGGAGAAA	GAGGCTGAAG	ATGCTTCAAG	TAACGAAGAC	GATGTGGACG	AGATTTACGT	1440		
CAAGAATGCT	TCTCTCGTAT	TCAGAGCTCT	TTGCAAGATT	TTGCAATAAG	TCTCTAGCCA	CAGAGCAGAA	CAGATCAGCT	1520		
AGTCCAGAAA	TATGAGATCC	AAATTGCTAT	CTCTGCACCT	AATCCACTAC	CTCATCAATA	ACCACGTCAT	TATCTTCACC	1600		
ACACCCCTTC	TCACACTAAA	AAACAGCTCA	GGTAATTTGG	AAGCGATGAC	CTTCTTTCAA	GCGATTAGAC	CTCATCTCTG	1680		
TTTAAGTCTC	AGTCGAAACG	GCGCCAGCTC	AGTACCGAAA	GTCTTCGAGG	TCGTCTGCGA	AATATTTTGG	CTCATGTATA	1760		
AACACATGAG	AGTCTAGATG	AAGGTTCTGT	GTCTTCTGAA	TTGACTTTGG	TATGTCTAAC	CAGTAGCAGA	AAGAGTTAGA	1840		
GGTCTTTCATG	AAGGAAATAT	ATCTAGCCAT	ACTCGAGAAG	CGAAATGCAC	CGGCTTTTCA	AAAGCAATAT	TTCATGGAGA	1920		
TATTAGAGCG	ACTGGCAGAC	GAGCCTCGTG	CATTAGTCGA	GATGTATCTC	AATACGACT	GTGATCGCAC	TGCCCTGGAG	2000		
AAATATTTTC	AAAAGTTTGT	TCTCCATTTT	AGTCTGAGAA	CAGGAAGAAT	CTGACAAAT	TCGCAGTATC	ATTGAACAGT	2080		
TATCAGTACA	GCAGTAGTATC	CCGACAGCTG	TGAACCTTCT	CCACAACAA	CAATACCACG	AATTGCAATG	GGTCTACTTC	2160		
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ACCTACGCAT	AGTGTGCCCT	CAGAATACAT	CTTAAACAT	CAAGCTGTGC	AGTGCCTTGT	TGTGATCTGT	GAATCCTTGG	2320		
ACAACCTGGG	CTCACAACGT	AGCGTTGACC	CGACTGCCGC	CCGGACCTTT	TCACAGAAGT	CTGTTGACAA	CCCACGAGAT	2400		
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<u>AGTTCTCTGAT</u>	<u>GACGATCCAA</u>	<u>GCCAGGTAGA</u>	<u>GAAGGTCAAG</u>	<u>CAGAGAAAGA</u>	<u>TCGCGCTCAC</u>	<u>AAACGTGATC</u>	<u>CAACAGTTCA</u>	2560		
<u>ACTTCAAACC</u>	<u>CAAGCGAGGC</u>	<u>GTTAAGCTTG</u>	<u>CCCTCCAAGA</u>	<u>AGGCTTTCATA</u>	<u>CGGTCTGACT</u>	<u>CTCCCGAGGA</u>	<u>CATTGCAGCT</u>	2640		
<u>TTCATACTTC</u>	<u>GCAATGACCG</u>	<u>CTTAGATAAG</u>	<u>GCTATGATCG</u>	<u>GAGAATACCT</u>	<u>TGGTGAGGGC</u>	<u>GACGCCGAAA</u>	<u>ACATCGCTAC</u>	2720		
<u>CATGCACGAT</u>	<u>TTTGTGTATA</u>	<u>TGATCGACTT</u>	<u>TTCCAAGCGG</u>	<u>CGCTTCTCTG</u>	<u>ACGCCCTCCG</u>	<u>CTCATCTCTTA</u>	<u>CACACTTTCC</u>	2800		
<u>GCCTGCCCGG</u>	<u>AGAAGCCGAG</u>	<u>AAGATCGACC</u>	<u>GTTTCTATGCT</u>	<u>CAAATCTCTCT</u>	<u>GAGCGTTATG</u>	<u>TTACTCAAAA</u>	<u>TCCGAATGCA</u>	2880		
<u>TTTGCCAACG</u>	<u>CGGATACGGC</u>	<u>GTACGTGCTT</u>	<u>GCGTACTCTG</u>	<u>TTATCTCTGCT</u>	<u>TAACACCGAC</u>	<u>CAGCATAGCT</u>	<u>CCAAGATGAA</u>	2960		
<u>AGGCCGTCGC</u>	<u>ATGACTAAGG</u>	<u>AAGACTTCAT</u>	<u>CAAAAACAAC</u>	<u>CGTGGTATTA</u>	<u>ATGACAATCA</u>	<u>GGACTTGCTT</u>	<u>GATGAATATC</u>	3040		
<u>TTGGTTGCAAT</u>	<u>CTTCGACGAA</u>	<u>ATCGCAAAACA</u>	<u>ACGAGATTTG</u>	<u>CTTGGACACC</u>	<u>GAAGTAGAGC</u>	<u>AGACTGCAAA</u>	<u>TGCCGCCCAT</u>	3120		
<u>CCCGCACCTG</u>	<u>TTCCTAGCGG</u>	<u>TCTTGATCA</u>	<u>AGGGCTGGGC</u>	<u>AAGTCTTCGC</u>	<u>AACGGTTGGA</u>	<u>AGGGATATAC</u>	<u>AGGGCGAGAG</u>	3200		
<u>ATATGCGCAA</u>	<u>GCCTCTGAGG</u>	<u>AAATGGCCAA</u>	<u>CAAACTGAG</u>	<u>CAACTCTATC</u>	<u>GCAGTTTGAT</u>	<u>CCGAGCTCAA</u>	<u>CGGAAGACTG</u>	3280		
<u>CGGTGAAGA</u>	<u>GGCATTTC</u>	<u>CGCTTTATCT</u>	<u>TCGCAACGCT</u>	<u>CGTCCAGCAC</u>	<u>GCTGGGTCCA</u>	<u>TGTTCAATGT</u>	<u>TACTTTGGATG</u>	3360		
<u>TCATTTCTCT</u>	<u>CCGGCTTGTC</u>	<u>CGCACCCATG</u>	<u>CAGGATACCC</u>	<u>AAACCTTAA</u>	<u>ACAATCAAGC</u>	<u>TCTGTATGGA</u>	<u>AGGTATGAAA</u>	3440		
<u>TTAGCCATAC</u>	<u>GAATCAGCTG</u>	<u>TACGTTTGAT</u>	<u>TTGGAACACT</u>	<u>CGCGGGTTGC</u>	<u>CTTCGTCACT</u>	<u>GCCTTGCGCA</u>	<u>AGTTTACAAA</u>	3520		
<u>CCTGGGCAAT</u>	<u>GTGAGGAGAG</u>	<u>TGGTCGCAAA</u>	<u>GAATGTCGAG</u>	<u>GCAGTAAAGA</u>	<u>TACTGCTTGA</u>	<u>TGTCGCGCTT</u>	<u>TCAGAGGGGA</u>	3600		
<u>ACCATCTAAA</u>	<u>GAGCTCTTGG</u>	<u>AGAGATATCC</u>	<u>TCACATGCGT</u>	<u>CAGCCAGCTG</u>	<u>GACAGGCTTC</u>	<u>AACTGCTCAG</u>	<u>CGACGGGGTC</u>	3680		
<u>GACGAGGGAT</u>	<u>CCTTGCCAGA</u>	<u>TATGTCACGC</u>	<u>GCTGGCGTTG</u>	<u>TGCCTCCGTC</u>	<u>TGCGTCCGAT</u>	<u>GGCCCCAGAA</u>	<u>GGTCAATGCA</u>	3760		
<u>GGCACCAAGG</u>	<u>CGTCTCGCG</u>	<u>CCAAGTCCAT</u>	<u>CACAGGCCCT</u>	<u>ACACCTTTTC</u>	<u>GAGCCGAAAT</u>	<u>AGCAATGGAA</u>	<u>AGTCGCTCAA</u>	3840		
<u>CAGAGATGGT</u>	<u>GAAGGGTGTA</u>	<u>GACCGATCT</u>	<u>TTACCAACAC</u>	<u>AGCTAATTTG</u>	<u>TCCCACGAAG</u>	<u>CTATCATCGA</u>	<u>CTTCGTTCCG</u>	3920		
<u>GCTCTTAGTG</u>	<u>AAGTGAGTTG</u>	<u>GCAAGAGATT</u>	<u>CAGTCTTCTG</u>	<u>GCCAGACGGC</u>	<u>CTCTCCTCGC</u>	<u>ACTTATAGTT</u>	<u>TGCAGAAGTT</u>	4000		
<u>GGTTGAAATC</u>	<u>ATTTATTACA</u>	<u>ACATGACACG</u>	<u>AGTCAGAATT</u>	<u>GAATGGTCCA</u>	<u>AGATCTGGGA</u>	<u>AGTTCTCGGG</u>	<u>CAGCACTTTA</u>	4080		
<u>ACCAAGTTGG</u>	<u>ATGCCATTTC</u>	<u>AACACAACGG</u>	<u>TGGTTTTCTT</u>	<u>CGCGTTAGAT</u>	<u>TCACTCCGTC</u>	<u>AACTATCAAT</u>	<u>GCGTTTTATG</u>	4160		
<u>GAGATTGAAG</u>	<u>AACTACCAGG</u>	<u>ATTCAAATTC</u>	<u>CAGAAAGACT</u>	<u>TCCTCAAACC</u>	<u>GTTGAGCAT</u>	<u>GTGATGTCCA</u>	<u>ATAGCAATGC</u>	4240		
<u>TGTTACTGTA</u>	<u>AAGGACATGA</u>	<u>TCCTCCGTTG</u>	<u>CCTCATACAG</u>	<u>ATGATTCAGG</u>	<u>CTCGCGGAGA</u>	<u>CAACATCCGT</u>	<u>TCTGGTTGGA</u>	4320		
<u>AAACGATGTT</u>	<u>TGGTGTCTTC</u>	<u>TCGTTTGCGG</u>	<u>CTCGAGAGCC</u>	<u>TTATGGTAGG</u>	<u>TTTGGCACTA</u>	<u>CCAGCGCTTT</u>	<u>ATACAAACGAT</u>	4400		
<u>ATCGGCTAAT</u>	<u>GGCAGATACA</u>	<u>GAGGGCATTG</u>	<u>TGAATATGGC</u>	<u>ATTGAGGCAC</u>	<u>GTTACTCAGA</u>	<u>TCTACAACAC</u>	<u>CGCCTTCGGT</u>	4480		
<u>GTGCTTATTA</u>	<u>CGCAAGGAGC</u>	<u>CTTCCCTGAC</u>	<u>CTTGTGTAT</u>	<u>GCCTCACAGA</u>	<u>GTTCTCTAAG</u>	<u>AACACGAGAT</u>	<u>TCCAGAAGAA</u>	4560		
<u>GTCATACAG</u>	<u>GCAATCGAGC</u>	<u>TTCTAAAGTC</u>	<u>GACGGTCGCC</u>	<u>AAAATGCTGA</u>	<u>GGACTCCAGA</u>	<u>ATGTCTCTTA</u>	<u>TCTCACCGTA</u>	4640		
<u>GCTCTACGGA</u>	<u>AGCTTTCCAT</u>	<u>GAGGACTCCA</u>	<u>CTAACCTCAC</u>	<u>TCAGCAACTC</u>	<u>ACTAAGCAGT</u>	<u>CCAAAGAAGA</u>	<u>GCAATTTTGG</u>	4720		
<u>TATCTTATTT</u>	<u>GATTTGCGTT</u>	<u>CCAAGATATT</u>	<u>CTCATGACTG</u>	<u>GCGATGATCT</u>	<u>TGAGGCTCGG</u>	<u>TACCGGTAA</u>	<u>GCTCTTCGAT</u>	4800		
<u>TACAGTATTT</u>	<u>GGACTATTTA</u>	<u>CTAACTCCGA</u>	<u>ACACTAGGGC</u>	<u>CTTGACATAC</u>	<u>CTTTTCGACA</u>	<u>CCTTAATACG</u>	<u>TTACGGAGGT</u>	4880		
<u>AGCTTCCCTC</u>	<u>AAGAATTCTG</u>	<u>GGATGTCCCT</u>	<u>TGGAGGCAGC</u>	<u>TCCTGTATCC</u>	<u>CATCTTCGTT</u>	<u>GTTCTGCAGT</u>	<u>CCAAATCGGA</u>	4960		
AATGTCCAAG GTACC										
(KpnI) T7										

Fig. 3.7 pYY2 sequence. The putative Sec 7 domain coding region is underlined. *KpnI* cutting sites are at either end of pYY2 (bold).

3.3 Transposon-arrayed pYY2 disruption

Gene knock-out (replacing a gene of interest with a selectable marker in a strain deleted for that marker) is used to answer whether a novel gene is essential for growth, but it is time consuming in *A. nidulans*. Transposon-arrayed gene knock-out (TAGKO) has been used to create gene disruption cassettes for transformation and mutant analysis (Hamer *et al.* 2001). A concern with using gene disruption with a small insertion is that there may still be a functional gene product. The Tn1000 transposon is about 6 kb long and is sufficient to disrupt the expression of a gene when it is inserted into the essential part of a target (Broom *et al.* 1995). The set of clones of pYY2 used for sequencing with the Tn1000 insertion about 500 base pairs apart, were co-transformed to ASK166 with ARp1 (Fig. 3.8). Insertions in the non-Sec7-domain coding region were able to complement *hypB5* defective phenotype at restrictive temperature, whereas those with insertions in the putative Sec7 domain coding region could not. The latter transformants had characteristic *hypB5* restrictive growth phenotype on medium lacking pyrimidines after transformation showing that they were transformed with ARp1 (Fig. 3.8). This result shows that *hypB5* complementing activity resides in the Sec7 domain.

A



B

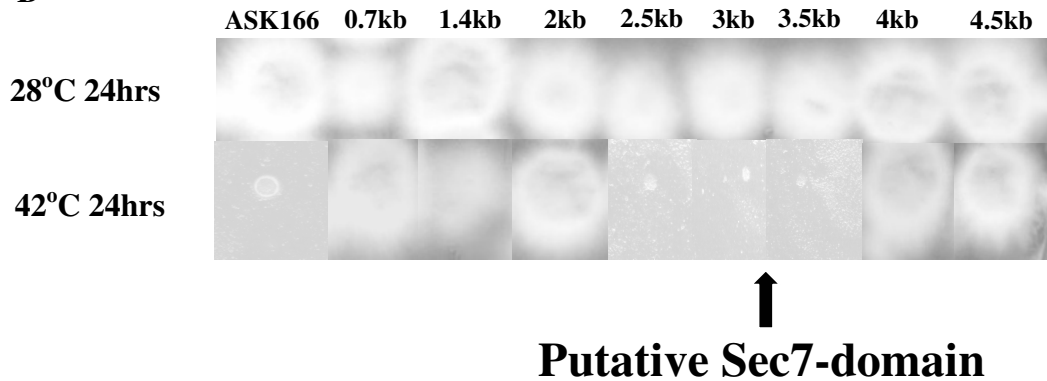


Fig. 3.8 Transposon-arrayed gene disruption of the *hypB5* complementing sequence using pYY2 with Tn1000 insertions. (A). Diagram of pYY2 disruption positions with Tn1000. The hatched box is the predicted Sec7 domain coding region. The filled triangles show Tn1000 insertions in the Sec7 domain, and open triangles show Tn1000 insertions elsewhere in pYY2 but not in the putative Sec7 domain. (B) Plate phenotype of ASK166 transformed with a panel of pYY2 clones with Tn1000 insertions about 500 bp apart. ASK166 and transformed colonies were grown at 28°C and 42°C for 24 hours. ASK166 was grown on medium containing pyrimidines whereas the transformed strains receiving ARp1 were selected on medium without pyrimidines. Successful *hypB5* complementation, implying the transposon disruption had not occurred in an essential region, was shown by strains with wild type growth at 42°C. Insertions into the predicted Sec7 domain failed to complement the *hypB5* phenotype.

3.4 Bioinformatic analysis of pYY2 sequence

3.4.1. pYY2 DNA sequence analysis

The pYY2 sequence was found to be on chromosome I using the recently published *A. nidulans* genome (www-genome.wi.mit.edu/annotation/fungi/aspergillus). The location of pYY2 is different from that of the *hypB* locus, which has been mapped on the right end of chromosome VII using both meiotic and mitotic gene mapping approaches (Kaminskyj and Hamer, 1998 and Kaminskyj, unpublished). This discrepancy will be discussed in section 4.3. Using Genscan (<http://genes.mit.edu/GENSCAN.html>), the exon regions of pYY2 were predicted (Fig. 3.9). pYY2 appears to contain a partial gene coding region lacking its 3' end. The highest Genscan prediction score, >100, which indicates a strong likelihood that the prediction will be valid, is in exon 2 which encompasses the gene disruption clones that failed to complement *hypB5*. The missing 3' end sequence downstream of pYY2 was retrieved from *A. nidulans* genomic database. Genscan analysis with the retrieved sequence predicted that the stop codon of the gene was about 1.3 kb downstream of pYY2 (Fig. 3.9). The entire retrieved gene sequence has homology to Sec7 domain proteins by using BLASTX (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Thus the retrieved entire gene sequence was designated as *A. nidulans* Sec7. There are four copies of the Sec7-domain-like sequences in *A. nidulans* genome. Two of them are on chromosome I, the others are on chromosome VI and VIII.

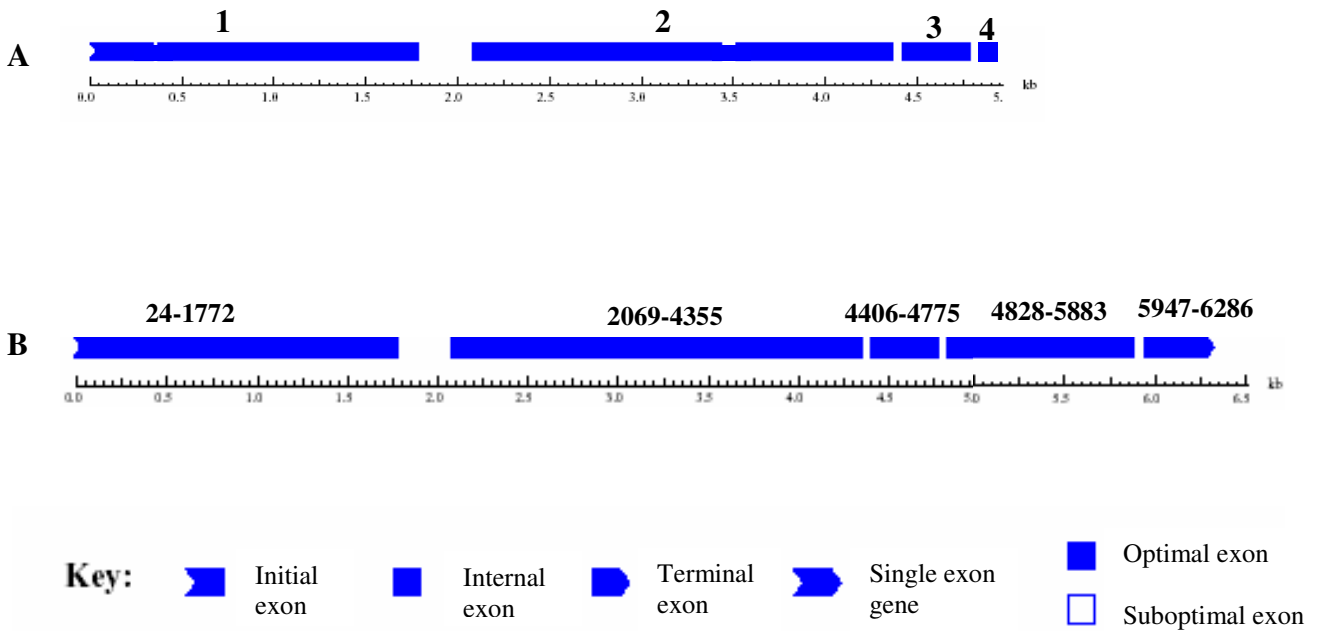


Fig. 3.9 (A) Analysis of the pYY2 sequence with Genscan

(<http://genes.mit.edu/GENSCAN.html>). The putative exons of pYY2 were predicted and shown as the bars above the sequence length scale. pYY2 appears to contain a partial gene coding region lacking its 3' end. Exon numbering is above the exon bars. Exon 2 has the highest Genscan prediction score. (B) Genscan analysis of *A. nidulans* Sec7. The start and end numbering (derived from pYY2 numbering) of individual exons is above each exon bar. The predicted stop codon of the gene is about 1.3 kb downstream of pYY2.

3.4.2 Analysis of predicted Sec7 domain of *A. nidulans* Sec7 protein

The amino acid sequence of the predicted protein encoded by *A. nidulans* Sec7 is shown in Fig. 3.10. *A. nidulans* Sec7 protein has 1933 amino acids, with the molecular weight of 219.1 kD. The predicted Sec7 domain consists of 190 amino acids (underlined), with an expected molecular weight of 21.9 kD (http://ca.expasy.org/tools/pi_tool.html).

```
MAEAENDPTNELNQTSVTQADKQNGVDVATEPHAPEVVAESKPALTDESERQEIPTIKEN 60
EDTMANNRLNDSKNNLPHEPSVTSPDTTTTDSNEPTDEPEQPHTEGDQLETLQDDQPPASD 120
EQLNEAPDAPSTRDEQLAQDMRQRSDSRSTTATFATNRSSVVSSTVFIVTALDAIGASRE 180
ARKSKELEDVAVKNALANVKQSDRQPIDPEILFYPLLLASRTLSPQLQVTALDCIGKLITY 240
SYFAFPSPAQEAKPSEADATAAEQPPLIERAIDAICDCFENEATPIEIQQQIIKSLLAAVLN 300
DKIVVHGAGLLKAVRQIYNMFIYSKSSQNQQIAQGSILTQMVSTVFDRLRVRLDLRELIR 360
EGEKAQAGSSESVTIEPVVSPPSAEDDQASDVASVAADQPVSKPEKLTLESFESNKDV 420
TTVNDNVPTMVTRANINQKRTQSYSGTSSEEKEAEDASSNEDDVDEIYVKDAFLVFRALC 480
KLSHKVLSEHQQDLKSQNMRSKLLSLHLIHYLINNHVIIFTTPLLTLKNSSGNLEAMTF 540
LQAIRPHLCLSLSRNGASSVPKVFECCEIFWLMLKHMRVMMKLSRYASIPTVVNPLQQQ 600
QYHELHVKASSVGNEWHQRGTLPPNLTSASIGNNQPPHTSVSEYILKHQAVECLVIL 660
ESLDNWAQSRSVDPTAARTFSQKSVDNPRDSMDSSAPAFLASPRVDGADGSTGRSTPVPD 720
DDPSQVEKVKQRKIALTNVIQQFNFKPKRGVKLALQEGFIRSDSPEDIAAFILRNDRLDK 780
AMIGEYLGEFDAENIATMHAFVDMMDFSKRRFVDALRSFLQHFRLPGEAQKIDRFMLKFS 840
ERYVTQNPANAFANADTAYVLAYSVILLNTDQHSSKMKGRRMTKEDFIKNNRGINDNQDLP 900
DEYLGSI FDEIANNEIVLDTEREQAANAHPAPVPSGLASRAGQVFATVGRDIQGERYAO 960
ASEEMANKTEQLYRSLIRAQRKTAVKEALSRFIFATSVQHAGSMFNVTWMSFLSGLSAPM 1020
QDTQNLKTIKLCMEGMKLAIRISCTFDLETPRVAFVTALAKFTNLGNVREMVAKNVEAVK 1080
ILLDVALSEGNHLKSSWRDILTCVSQDLRLQLLSDGVDEGLPDMSRAGVVPPSASDGPR 1140
RSMQAPRRPRPKSITGPTPFRAEIAMESRSTEMVKGVDRIFTNTANLSHEAII DFVRALS 1200
EVSWQEIQSSGQTASPRTYSLQKLVEISYYNMTRVRIEWSKIWEVLGQHFNQVGCHSNTT 1260
VVFALDSLRLQSLMRFMEIEELPGFKFKQDKFLKPFHVMSNSNAVTVKDMILRCLIQMIQ 1320
ARGDNIRSGWKTMTFGVFSFAAREPYDTEGIVNMAFEHVTQIYNTRFGVVITQGAFFDLVV 1380
CLTEFSKNTRFQKKSLQAIELLKSTVAKMLRTPCPLSHRSSTEAFHEDSTNLTOQLTKQ 1440
SKEEQFWYPILIAFQDILMTGDDLEARSALTYLFDTLIRYGGSFPPQEFWDVLWRQLLYP 1500
IFVVLQSKSEMSKVPNHEELSVWLSTTMIQALRHMITLFTHYFDALEYMLGRVLELLTLC 1560
ICQENDTIARIGSNCLQQLILQNVKFKQKDHWNKTVGAFIELFNKTTAYELFTAATTMAT 1620
VTLKTPSAPTANGQLADTHDTVQDPTESSPAQETSTEPKLNQGTQDTTAEHEDGDMPAAS 1680
NTELEDYRPQSDTQQQPAAVTAARRRYFNRIITSCVLQLLMIETVHELFSNDKVYAQIPS 1740
HELLRLMGLLKSYQFAKKFNEDKELRMQLWRQGFMKSPPNLLKQESGSAATYVHILFRM 1800
YHDEREERKSSRSETEAALIPLCVDIISGFVRLDEDSQHRNIVAWRPVVVDVIEGYTNFP 1860
AEGFDKHIDTFYPLAVDLLGRELNSEIRLAIQGLFQRIGEARLGLPVRPTPTPVSPRHSV 1920
SEHPSRKHSVGRR
```

Fig. 3.10 The amino acid sequence of the predicted Sec7 protein encoded by *A. nidulans* Sec7. The Sec7 domain is underlined.

The Sec7 domains are highly conserved from yeast to mammals (www.ncbi.nlm.nih.gov/blast), including *Saccharomyces cerevisiae* (Goffeau *et al.* 1996), *Schizosaccharomyces pombe* (Wood *et al.* 2002), *Neurospora crassa* (Galagan *et al.* 2003), *Homo sapiens* (www.ncbi.nlm.nih.gov/blast), *Mus musculus* (www.ncbi.nlm.nih.gov/blast) and *Caenorhabditis elegans* (www.ncbi.nlm.nih.gov/blast). The *A. nidulans* pYY2 predicted Sec7 domain is 81% identical to that of *S. cerevisiae*. The alignment of the pYY2 Sec7 domain and other Sec7 domains is shown in Fig.3.11. The Sec7 domain encoded by pYY2 likely has 10 alpha helices. The predicted secondary structure is 60.9 % alpha helix and 39.1 % coil (www.bork.embl-heidelberg.de/cgi/sscp_serv.pl). The pYY2 Sec7 domain tertiary structure was predicted and mapped against a model of the human Arf nucleotide binding openner (ARNO) Sec7 domain (<http://www.ch.embnet.org/software/BottomBLAST.html>) homologue by using the software MODELLER (Šali and Blundell, 1993) as shown in Fig. 3.12, with the help of Dr. David Sanders, Chemistry.

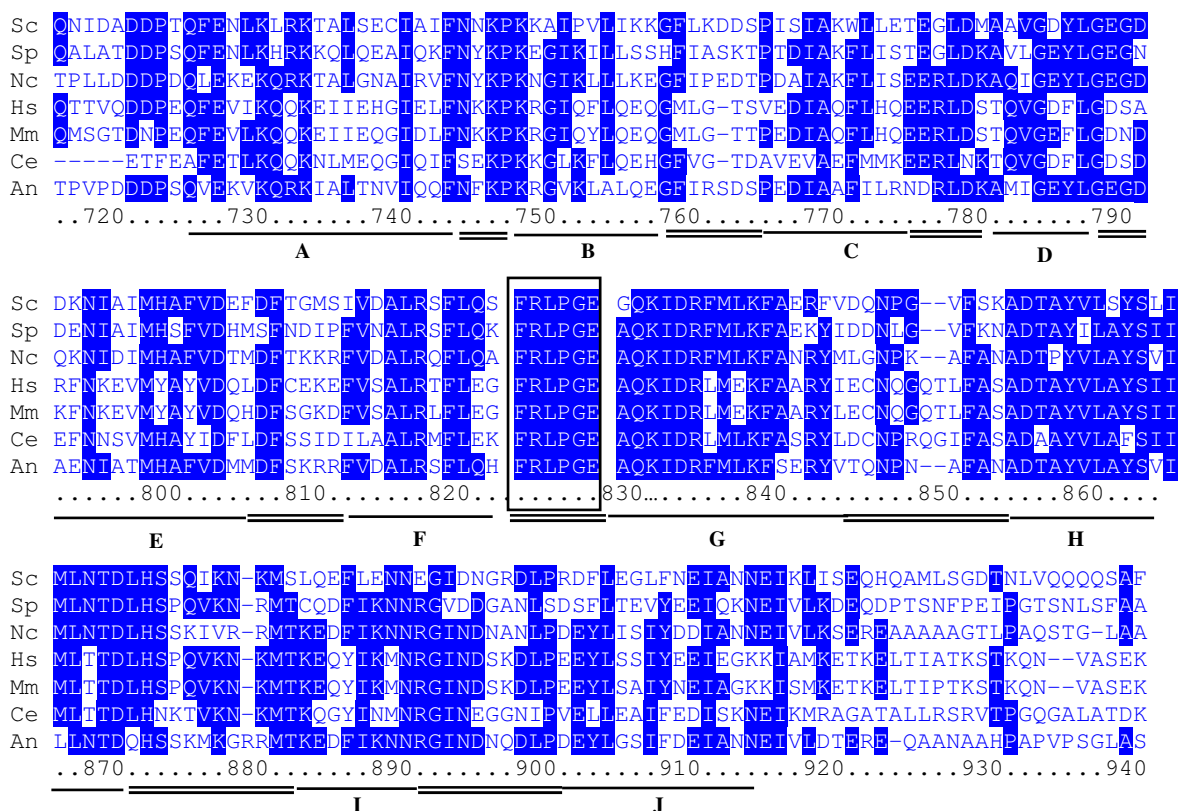


Fig. 3.11 Sequence alignment of predicted Sec7 domains from selected fungi and animals (Sc= *Saccharomyces cerevisiae*, Sp= *Schizosaccharomyces pombe*, Nc= *Neurospora crassa*, Hs= *Homo sapiens*, Mm= *Mus musculus*, Ce= *Caenorhabditis elegans*, An= *Aspergillus nidulans*). Identical residues are box-shaded. The secondary structure of Sec7 domain encoded by pYY2 was predicted with www.bork.embl-heidelberg.de/cgi/sscp_serv.pl. Predicted α -helix regions are single underlined (A-J), coils are double underlined. The FRLPGE motif (framed residues, *A. nidulans* Sec7 protein numbering 823-828) has been shown to be important for GEF activity in human ARNO.

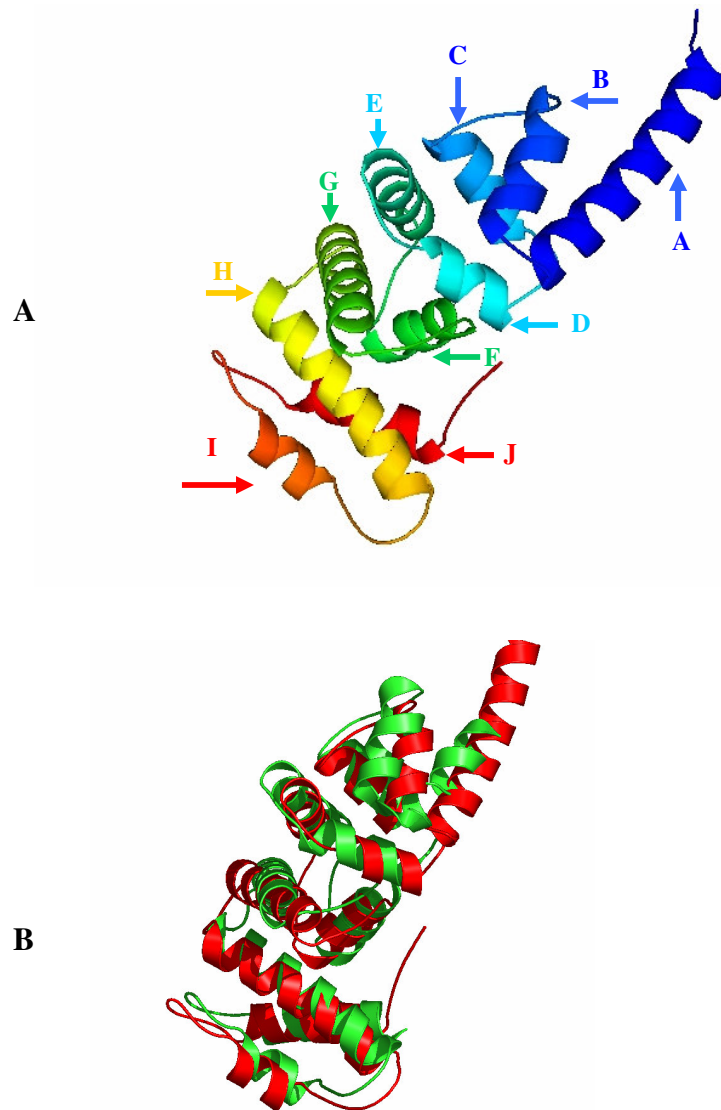


Fig. 3.12 (A). Ribbon representation of human ARNO Sec 7 domain. The Sec7 domain exclusively consists of ten α -helices, the deep hydrophobic groove comprising α -helices F, G and H. The F-G loop is the most highly conserved motif. (B). Modeling of pYY2 Sec7 domain against ARNO crystal structure. The red structure is human ARNO Sec7 domain crystal structure (<http://www.ch.embnet.org/software/BottomBLAST.html>). The green structure is the predicted pYY2 protein Sec7 domain model based on MODELLER.

3.5 Mating of *hypB5* with *podB1*

podB1 is an *A. nidulans* morphogenetic mutant isolated by Harris *et al.* (1999) from the same collection as Harris *et al.* (1994). *podB1* is involved in polarity establishment, *podB* may also be required for the organization of the cytoskeleton at the extreme tip (Harris *et al.* 1999). At the restrictive temperature, *podB1* spores accumulate more than 8 nuclei per cell before germinating, which suggests this strain has delayed polarization. *podB1* germling upshifted from 28°C to 42°C leads to the cessation of tip growth. Thus the *podB1* restrictive phenotype somewhat resembles that of *hypB5*. Like *hypB*, *podB* has been cloned and found to be in a pathway that is distinct from that involving *hypA* (= *podA*). It was of interest to determine if *hypB* and *podB* were allelic. In order to make a *hypB5*, *podB1* double mutant, ASK166 (*pyrG89*; *hypB5*; *wA2*; *veA1*) was mated with ASH83 (*pabaA6*; *podB1*; *pryoA4*; *veA1*). Although outcrossed cleistothecia were obtained, I could not isolate the double mutant, suggesting that these alleles are synthetically lethal, also they are not allelic.

3.6 Identifying the *hypB5* strain Sec7 domain lesion

Transposon-arrayed gene disruption demonstrated that pYY2 was no longer able to complement *hypB5* defects with Tn1000 insertions within Sec7 domain coding region. The abnormal phenotype of *hypB5* may be caused by the loss of Sec7 domain function which plays an important role involving in intracellular vesicle trafficking by inducing the conformational exchange of ARF that initiates COPI vesicle budding. The *hypB5* strain Sec7 domain lesion was expected to be located at the highly conserved F-G loop, α -helix H or the components of the deep hydrophobic groove which are the critical sites for the nucleotide exchange activities. pYY2 Sec7 domain disruption analysis and the structure of human Sec7 protein ARNO focused the search for *hypB5* strain Sec7 domain lesion, PCR primers were designed to amplify the putative Sec7 domain coding region from ASK166 genomic DNA (Fig. 3.13).

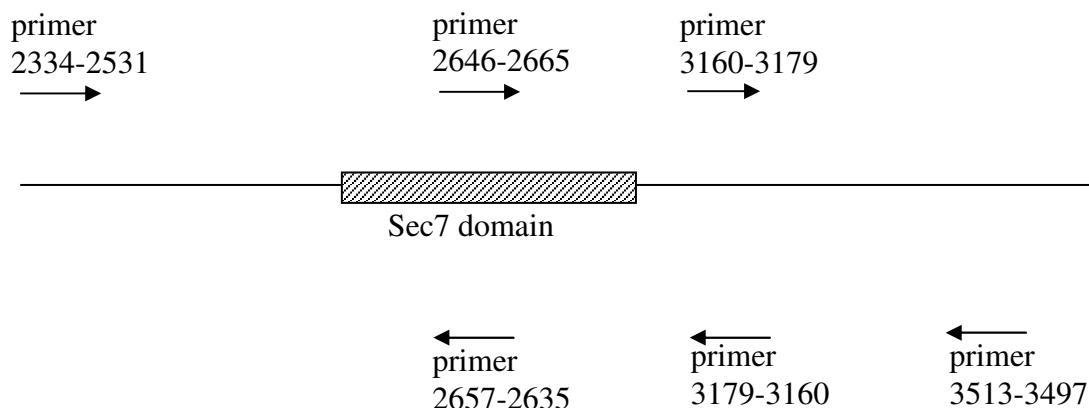
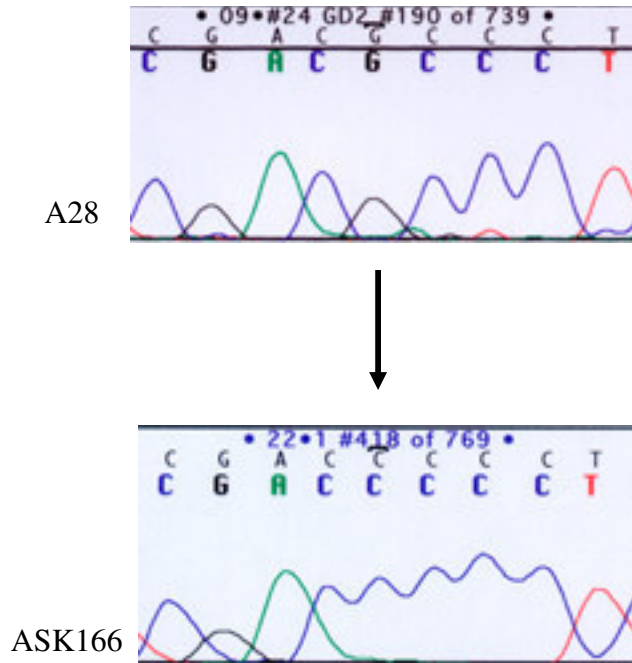


Fig. 3.13 The primers designed for amplification and sequencing of ASK166 putative Sec7 domain coding region. Numbering refers to pYY2 sequence shown in Fig. 3.7.

Primers designed to amplify the *A. nidulans* Sec7 domain coding region are listed in Table 2.3. First round of PCR with primers pYY2 2334-2531 and pYY2 3513-3497 created two products: the expected 1.2 kb band, and a 300 bp band. For sequencing, the 1.2 kb fragment was isolated by gel electrophoresis and used as the template for the seminested PCR employing primer pYY2 2334-2531 and primer pYY2 3179-3160.

Comparison of *hypB5* strain Sec7 domain coding region sequence with the wild type counterpart showed that there was a G substituted with a C at location 2773 (pYY2 numbering) as shown in Fig. 3.14. As a result, an A815P (*A. nidulans* Sec7 protein numbering) occurred in Sec7 domain α -helix F (Fig. 3.14 and 3.15).

A



B

Sc	IVDALRSFLQS
Sp	FVNALRSFLQK
Nc	FVDALRQFLQA
Hs	FVSALRTFLEG
Mm	FVSALRLFLEG
Ce	ILAAALRMFLEK
(A28) An	FVDALRSFLQH
(ASK166) An	FVDPLRSFLQH

*

Sec7 domain alpha helix F

Fig. 3.14 Comparison of the Sec7 domain DNA sequences for ASK166 (*hypB5*) and A28 (wild type, ASK166 parental strain) shows a G to C substitution (panel A). As a result, an amino acid residue alanine was replaced with proline within the α -helix F (panel B), indicated by an asterisk.

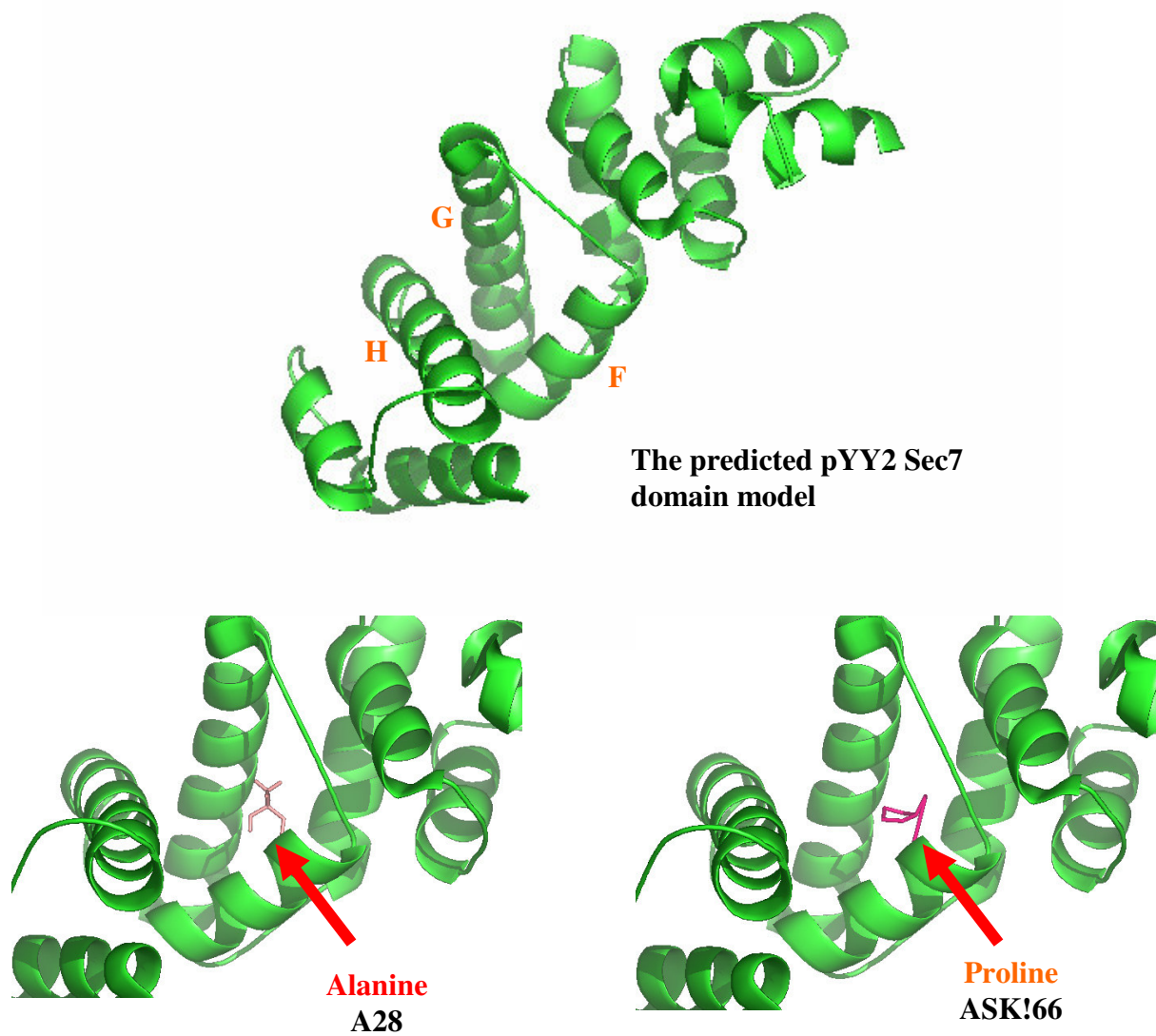


Fig. 3.15 The top panel is the predicted pYY2 Sec7 domain model rotated from Fig. 3.12 to show clearly the residue substitution. α helices F, G and H are indicated. The left one in the lower panel shows an alanine in the Sec7 domain α helices F of A28, whereas it is a proline of ASK166 indicated in the right picture of the lower panel

3.7 pYY2 cDNA

A. nidulans partial cDNA sequences are available from the website:

<http://www.genome.ou.edu/fungal.html>. The pYY2 cDNA was not included since the pYY2 sequence could not retrieve its expressed sequence tag (EST) data. An *A. nidulans* cDNA was synthesized and used as a PCR template for isolating pYY2 cDNA

Primers were designed against the putative cDNA coding region (primer sequence see Table 2.3). If the primers were located within an exon region, a PCR reaction with cDNA as template should show an amplification product. Sequencing these PCR products could help to define the pYY2 cDNA sequence, particularly intron boundaries. Unfortunately, after trials of different PCR buffer conditions, annealing temperatures and *Taq* or *pfx* DNA polymerases, only the pair of primers pYY2 2646-2665 and pYY2 3179-3160 generated an amplification band.

pYY2 cDNA boundaries and introns are still undefined. The primers pYY2 2646-2665 and pYY2 3179-3160 were located at the most highly conserved region encoding the Sec7 domain. PCR product from amplification with cDNA as template supported the notion that the pYY2 sequence may participate in regulating hyphal morphogenesis through its Sec7 domain function. No intron was detected in the pYY2 region from 2646 to 3179 PCR product. Rapid amplification of cDNA end (RACE) will be required to detect pYY2 cDNA 5' and 3' ends and its intron boundaries.

4. DISCUSSION

This study describes a Sec7 domain protein from *Aspergillus nidulans* that was cloned by complementing the *hypB5* temperature sensitive morphogenetic defect, which is due to a single gene mutation (Kaminskyj and Hamer, 1998). Complemented *hypB5* strains have wild type colony morphology. Sequence comparison using BLASTX shows that this Sec7 domain protein likely encodes an ADP-ribosylation factor guanine nucleotide exchange factor (ARF-GEF). Functional ARF-GEF is required for an early stage in COPI-dependent vesicle budding, which is consistent with the *hypB5* morphogenetic defect. An alanine replaced by a proline, the non-conservative amino acid change, was found in a conserved region of Sec7 domain of this putative ARF-GEF in the untransformed *hypB5* strain, ASK166, when compared to the R153 strain used to create the pRG3-AMA1 genomic library used in cloning. The strain used to create the *A. nidulans* genome database FGSC-4A, and A28 (the ASK166 mutagenesis parent strain) do not have this nucleotide substitution. This mutation could account for the temperature sensitivity of the *hypB5* allele.

Initially, it appeared that this was a straightward result. However, the recently released *A. nidulans* genome shows this putative ARF-GEF sequence is located on chromosome I, whereas the *hypB* locus is on chromosome VII. Morphometry of wild type A28, transformed AYY1 and *hypB5* ASK166 cells reveals subtle differences between the wild type and complemented strains. This suggests that the putative ARF-GEF sequence is a multicopy suppressor of the *hypB5* allele. Future research needed to fully characterize the Sec7 domain protein function and its relationship to *hypB5* will be discussed.

4.1 Cloning and sequencing the *hypB5* complementing DNA

Phenotypic complementation of fungal genes has provided an effective cloning approach particularly for *A. nidulans*, which has many well-characterized mutant strains. The strategy selected for cloning a given gene depends on the tools available. For

example, a precisely mapped gene could be cloned with a chromosome specific cosmid library (Kaminskyj and Hamer, 1998) but it would be time consuming if the gene locus is unknown. Digestion of genomic DNA for construction of a cosmid library may cut the gene of interest and lead to the gene distributed on adjacent cosmids. Alternatively, a genomic library with overlapping fragment inserts from partial restrictive enzyme digestion provides a convenient way to identify a complementing clone: the library should have multiple genome coverage and can be probed in a single transformation. After transformation, colonies with wild type features are identified and the corresponding plasmid bearing the gene of interest can be rescued into *E. coli*. By digesting the rescued corresponding plasmid with a panel of restriction endonucleases, the smallest fragment DNA which is able to recover wild type phenotype can be selected for subcloning. This procedure was successfully applied to clone genes in *A. nidulans* (e.g. Oshero *et al.* 2000a, b). However, a multi-copy extragenic suppressor gene rather than the gene of interest may be isolated if the entire genome is tested simultaneously. This is particularly likely when an AMA1 bearing plasmid is used to increase fungal transformation efficiency (Aleksenko and Clutterbuck, 1997). AMA1 is the only genomic region of *A. nidulans* acting as a plasmid replication origin. Transformation with the help of AMA1 sequence creates transformants that are stable under selective conditions, due to the autonomous replication feature of AMA1, but not chromosomal integrants (Aleksenko and Clutterbuck, 1997). However since AMA1 plasmids are typically found in multiple copies per fungal nucleus, it can also function as an overexpression vector.

In this study, the pRG3-AMA1 *A. nidulans* genomic library was used for *hypB5* complementation by phenotype rescue. pYY1 was isolated from this library by complementing the *hypB5* defective phenotype to give wild type colony growth at the restrictive temperature (Fig. 3.1). Unlike the parental strain, ASK166, the plate phenotype of *A. nidulans* strain AYY1, containing pYY1, resembled wild type at restrictive temperature (Fig. 3.2 and 3.3). However the cellular features of AYY1 did not resemble wild type entirely in all characteristics. At both permissive and restrictive temperatures, wild type A28 basal cells were longer and wider than those of AYY1, and AYY1 had on average more nuclei per basal cell than wild type (Table 3.1). The increased length of

wild type compared to AYY1 cells could have been due to procedural factors. The first few septa in a germling are more closely spaced than those deposited later (Kaminskyj, unpublished). However in wild type strains, the number of nuclei per basal cell is positively correlated with cell length, so it is problematic to explain how the transformed strain could have shorter cells with more nuclei.

Transposon-facilitated DNA sequencing was used in this study to create general priming sites. The reverse repeats, used as primer GDIR binding sites, at either end of Tn1000 provide a convenient way to map the transposition insertion site. Subsequently, the subterminal sequences adjacent to GDIR were used as sequencing primers GD1 and GD2 binding sites. Since the Tn1000 insertion occurs randomly in the target DNA fragment, a single round of mating can provide sufficient insertions evenly spaced along the target DNA. Appropriately spaced insertions screened by PCR can be used for sequencing simultaneously and bidirectionally outflanking Tn1000. For example, a 1 kb DNA fragment only requires one appropriate insertion for sequencing from both directions. Due to the large size of Tn1000, the insertion is also able to disrupt target gene function when it occurs at the essential part of that gene. Thus, this insertion strategy can not only be used for DNA sequencing but also for gene disruption analysis.

4.2 Does pYY2 contain the *hypB* gene or a multicopy suppressor of the *hypB5* defect?

The recently published *A. nidulans* genome (<http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/>) was queried using pYY2 sequence. pYY2 sequence was on contig No. 112 and supercontig No. 8 which was located on chromosome I. Sequence analysis with BLASTX indicated that pYY2 may encode a Sec7 domain containing protein. There were another three copies of the Sec7 domain coding sequence arranged along the whole genome. They were on linkage groups I, VI and VIII, but none was on chromosome VII, the location of the *hypB* locus.

The *hypB* gene has been mapped to chromosome VII using mitotic recombination (Kaminskyj and Hamer, 1998) and meiotic recombination with anchored markers on chromosome VII. Another anchored chromosome VII marker, *hbrA* was found to be closely linked with *hypB* with 1.4% recombination. The cosmid W01H09 used to clone

hbrA was not able to complement the *hypB5* mutant phenotype. However, *hypB5* complementation using neighboring cosmids was not successful, and because complementation using a cosmid plus ARp1 frequently causes genetic recombination, so that the chimaeric complementing plasmid can not be rescued intact into *E. coli* a single putative transformation (Shea, 1999). The distinct genetic localization of *hypB* and pYY2 and the variation in cell morphometry of A28 and AYY1 raise the question: does pYY2 contain the *hypB* gene or an extragenic suppressor?

Evidence suggesting pYY2 contains a multicopy suppressor of *hypB5* include: the pYY2 location is on chromosome I, whereas *hypB* is on chromosome VII. The pYY2 DNA sequence was unique amongst the whole genome, so it can not also be repeated in *hypB* on the right end of chromosome VII. The cellular characterization of the transformed strain AYY1 showed that at 42°C AYY1 had shorter and narrower subapical cells but more nuclei per basal cell than wild type. Nevertheless, the AYY1 colony and cellular phenotypes were quite different from those of its parent strain ASK166. If pYY2 contains *hypB* then transformants like AYY1 should not show significant difference from wild type. However since pYY2 does not contain the whole gene, there might be a missing critical region necessary for full phenotypic complementation. Since pYY2 did not contain the entire gene therefore this may help explain why the AYY1 strain exhibits cellular differences from wild type. Once the pYY2 cDNA is cloned, full complementation may be possible.

However, analysis of pYY2 conflicts with the interpretation that pYY2 contains a suppressor of *hypB5*. The Sec7 domain sequence amplified from ASK166 genomic DNA had a single base pair substitution, which led to non-conservative amino acid substitution at Sec7 domain α helix F, a critical component of a functional hydrophobic ARF binding groove. This amino acid substitution may cause *hypB5* temperature sensitivity in a temperature dependent manner. At permissive temperature, even in α -helix F of Sec7 domain bearing an A to P exchange, its Sec7 domain function may be maintained due to the intact Sec7 domain structure (Dr. Sanders, personal communication). But at restrictive temperature, the mutant Sec7 hydrophobic cleft may be less stable due to the residue exchange (discussed below). This analysis somewhat conflicts with the notion

that *hypB5* was a single gene mutation. If pYY2 does not contain *hypB*, which means except Sec7 point mutation, there should be another mutated site located in the *hypB* region. But *hypB5* mutant was generated with 4-NQO mutagenesis which usually generates single gene mutation (Bos, 1996). Crossing *hypB5* with GR5 (*pyrG89; pyro4; wA3*) and AH12 (*argB2; chaA*) showed 21:27 and 40:60 temperature sensitive segregation respectively, which is consistent with *hypB5* being a single gene mutation (Kaminskyj and Hamer, 1998). Had it been otherwise, it would be apparent during meiotic segregation mapping. In addition, a preliminary study of cloning *hypB* using the same genomic library also found the 5 kb *KpnI* fragment with *hypB5* complementing activity (Potlakayala and Kaminskyj, unpublished). A 5 kb *KpnI* fragment, the same size as the *KpnI* fragment in pYY2, was isolated independently from that previous study.

4.3 Analysis of pYY2

4.3.1 pYY2 encodes a Sec7 domain protein

Bioinformatic analysis suggests that pYY2 encodes a partial Sec7 domain protein (<http://ca.expasy.org/cgi-bin/scanprosite>). This predicted Sec7 domain has 81% identity to that of *S. cerevisiae* ARF-dependent Sec7 domain. The Sec7 domain is the only region conserved amongst all Sec7 proteins known so far which include *S. cerevisiae* (Goffeau *et al.* 1996), *S. pombe* (Wood *et al.* 2000), *Homo sapiens* (www.ncbi.nlm.nih.gov/blast). The Sec7 domain, which has been studied in human Arf nucleotide binding site opener (ARNO) encodes a guanine nucleotide exchange factor (Chardin *et al.* 1996). Sec7 domain is capable of exchanging GTP for GDP on ARF. ARF-GTP is required to initiate COPI vesicle formation (Serafini *et al.* 1991), thereafter contributing to intracellular vesicle trafficking. The Sec7 domain GEFs are exclusively α -helical proteins (Mossessova *et al.* 1998) which is consistent with the secondary structure prediction of pYY2 Sec7 domain (Fig. 3.11). The conserved hydrophobic residues are located at the central area extending along ten α -helices, named A-J (Cherfils *et al.* 1998). The prominent feature of the domain is the presence of a deep hydrophobic groove (comprising α -helices F, G and H) which acts as the nucleotide free ARF binding site. The most highly conserved F-G loop FRLPGE is also predicted in pYY2 Sec7 domain

(Fig. 3.11 frame box). Another conserved motif encompasses α -helix H. Point mutations within these conserved motifs in ARNO dramatically reduce the exchange activity, suggestion that these regions constitute the functional site of the Sec7 domain (Mossessova *et al.* 1998, Cherfils *et al.* 1998). Polarized delivery of cell wall and membrane building vesicles plays a critical role in morphogenetic regulation of fungal tip growth. Thus the pYY2 product may participate in tip growth via its Sec7 domain function. Tn1000 tagged Sec7 domain disruption showed that insertions within the Sec7 domain coding region disrupted pYY2 complementation activity but Tn1000 inserted outside the Sec7 domain coding region did not (Fig. 3.8). This result agreed with that of Chardin *et al.* (1996) who found that the Sec7 domain function alone is sufficient for the nucleotide exchange activity. Even though there are three other copies of the Sec7 domain coding sequence in the *A. nidulans* genome, pYY2 Sec7 domain shares the highest similarity with that of *S. cerevisiae*. Since *hypB5* strain must have wild type copies of the other three Sec7 domain coding sequences, it was likely that only the pYY2 Sec7 domain but not the other three copies of Sec7 domain coding region could complement *hypB5* defects, this prediction need to be proved individually.

The retrieved *A. nidulans* Sec7 sequence was used to search against Genbank. The downstream sequence of pYY2 also recognized Sec7 domain proteins in BLASTX. Without the pYY2 cDNA, it is unclear whether pYY2 encompasses both ends of this Sec7 coding region. Thus *A. nidulans* Sec7 protein coding sequence appeared to be larger than pYY2. Isolating the cDNA corresponding to pYY2 can provide further evidence supporting or correcting the computational prediction. Unfortunately only one pair of primers which were located within the most highly conserved region could amplify from A28 cDNA. Other primer combinations did not give rise to the expected results. Thus pYY2 introns, 5' and 3' ends are still unknown.

4.3.2 The *hypB5* strain has a lesion in its Sec7 domain

The *hypB5* strain may contain a lesion in its Sec7 domain coding region as suggested by: 1) pYY2 sequence contains a Sec7 domain coding region, 2) pYY2 Tn1000 disruption indicated that Sec7 domain function was necessary to complement

hypB5 temperature sensitive defect, 3) Sec7 domain alone is sufficient for the nucleotide exchange activity (Chardin *et al.* 1996). To test this idea, primers around the Sec7 domain coding region were designed to amplify ASK166 genomic sequence for identification of the *hypB5* strain putative Sec7 domain lesion. It was expected that the lesion position should be in the location which affects the protein tertiary structure for its interaction with its effectors or regulators, which was supported by *hypB5* strain Sec7 domain lesion identification result (Fig. 3.14 and 3.15). The lesion is a single base pair substitution from a G to a C. Thus at the protein structure level, the mutated codon encoded a proline instead of an alanine. The key point of the substitution was that it took place at the Sec7 domain α -helix F, which is one of the components of the functional deep hydrophobic groove considered in *S. cerevisiae* to be the ARF binding site (Cherfils *et al.* 1998). Known as an α helix disruptor, proline is also able to reside within an α helix in a temperature dependent fashion. In terms of the thermodynamic Gibbs free energy equation $\Delta G = \Delta H - T\Delta S$, a reaction will be favored when ΔG is negative. ΔS could be negative in the reaction of forming a hydrophobic groove. Thus when the temperature is increased, ΔG will be more positive which in turn may increase difficulties to form the hydrophobic groove (Dr. Sanders, University of Saskatchewan, Chemistry, personal communication). As a result, the *hypB5* strain Sec7 domain function may be abolished at restrictive temperature which in turn may be involved in *hypB5* temperature sensitivity. Other base pair exchanges occurring out of the Sec7 domain coding region could not be ruled out so far. But, those substitutions out of the Sec7 domain or even just out of the deep hydrophobic groove are not likely to be responsible for the mutant phenotype (Cherfils *et al.* 1998).

4.4 Resolutions of the puzzle: does pYY2 contain *hypB* gene or its suppressor?

4.4.1 Repeat transformation of ASK166 with the *A. nidulans* genomic library and/or cosmid library

The shortcoming of this study is only the transformant AYY1 was isolated for further cloning and analysis. Since the first round of transformation showed several wild type growth colonies in each plate, if all of them had been isolated and analyzed, maybe

not only the suppressor *Sec7* gene but also the *hypB* gene and other *hypB* suppressor genes would be isolated as well. Based on the released *A. nidulans* genome database, it would be more productive, and each isolated gene could be assigned to its chromosomal region for bioinformatics analysis.

Transformation with the chromosomal specific *A. nidulans* cosmid library will provide more precise evidence supporting the cloned gene will be the gene of interest. However, it is necessary to circumvent the difficulty of rescuing the complementing DNA into *E. coli*. Preliminary results using this strategy were equivocal, yielding a single transformant and could not be repeated.

4.4.2 Knock out Sec7 domain

Gene knock out of the ARF-Sec7 domain from wild type *A. nidulans* may reveal if it is essential for growth. *S. cerevisiae* has four Sec7 domain genes: SEC7, GEA1, GEA2 and SYT1. Except for SYT1, all are essential (Jones *et al.* 1999). The strategy of gene knockout uses a constructed deletion cassette to replace one copy of that gene by homologues recombination in a diploid strain. The putative crossover is selected by expression of the selectable marker under selective condition. Haploidization of the putative crossover strain gives rise to progenies carrying a wild type copy of that gene or the deletion cassette. Whether the gene is essential can be determined by the viability ratio of the progenies. The deletion can be confirmed by PCR and/or Southern blot.

If Sec7 is not essential for growth in *A. nidulans*, observation of the phenotype of the null mutation may provide additional evidence that pYY2 contains *hypB* or its suppressor. If generation of an *A. nidulans* Sec7 null mutation in a wild type strain gives rise to the *hypB5* mutant phenotype, then pYY2 is likely to contain *hypB*. If the null mutation has an obviously distinct phenotype from *hypB5*, pYY2 probably contains *hypB5* suppressor.

4.4.3 Repair ASK166 Sec7 domain lesion and mutagenesis of pYY2

ASK166 contains a lesion at its Sec7 domain α helix F. It is straightforward to test if repair of this lesion could rescue the *hypB5* mutant phenotype. Site directed mutagenesis

(Sambrook and Russel, 2001) which converts the nucleotide C in ASK166 Sec7 domain lesion point back to the wild type G may identify if ASK166 is a single gene mutant. If the repair restores ASK166 phenotype to wild type, ASK166 Sec7 domain lesion is critical for its temperature sensitivity. But it will still be unclear if ASK166 contains a single mutant gene, since the recovered nucleotide will restore ASK166 Sec7 domain function which may suppress *hypB5*. If the repair generates a phenotype other than ASK166 but not wild type, ASK166 is probably a potential double mutant. Its phenotype may be a synergistic effect of *Sec7* and *hypB*. The Sec7 lesion repaired strain from ASK166 thus can be used for cloning and analysis of *hypB*. But it is unlikely that *hypB5* strain contains double mutant, since *hypB5* segregated ~1:1 in two rounds of backcrossing to wild type and so the morphogenetic defect was judged to be a single gene lesion.

Mutagenesis of pYY2 at the mapped lesion site in ASK166 will generate a “mutated” plasmid. If the co-transformation of this nucleotide mutation bearing plasmid with ARp1 to ASK166 can not rescue the mutant phenotype, the same as in the transposon disruption experiment, this suggests that ASK166 Sec7 domain lesion is responsible for ASK166 phenotypic defects. If the “mutated” plasmid maintains complementation activity, it is not the Sec7 domain nucleotide exchange leading to ASK166 temperature sensitive phenotype but the unidentified *hypB5* lesion. Also ASK166 is a single gene mutant at its *hypB5* locus but not the silent Sec7 domain site.

Taken together, it appears more likely that pYY2 contains an extragenic *hypB5* suppressor than *hypB* itself. The final resolution of this puzzle will require the tests described above.

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